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TITLE: Use of Telemorace Inhibition in Combination with Anti-Cancer Drugs to Induce Cell Death in Tumor Cells

PRINCIPLE INVESTIGATOR: Maria Antonietta Cerone

CONTRACTING ORGANIZATION: S. M. B. D. – Jewish General Hospital
Montreal, Quebec, Canada H3T 1E2

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14. ABSTRACT
Telomerase is a ribonucleoprotein complex that maintains the stability of chromosome ends, the telomeres, and regulates cell replicative potential. The enzyme minimally contains a catalytic subunit with reverse transcriptase activity (hTERT) and a RNA subunit (hTR) with a region complementary to the telomeric repeats that is used as template. Telomerase is up-regulated in 95% of breast carcinoma, but not in adjacent normal tissues and its activity increases with tumor aggressiveness. Therefore targeting telomerase may represent a promising approach for cancer therapy. Inhibition of telomerase would result in telomere shortening and cell death due to dysfunctional telomeres. The major limitation of this approach is the time necessary for the telomeres to shorten sufficiently to engage cell death. One possibility to overcome this lag phase is to target the telomeres by introducing hTRs with mutations in the template region, which results in decreased cell viability and increased apoptosis. The aim of this study is to investigate the feasibility of a new anti-cancer approach based on the combination of telomere disturbances induced by mutant hTR and chemotherapeutic drugs. Our results show that interfering with telomere maintenance in breast cancer cells results in increased susceptibility to anti-cancer drugs independently of initial telomere length and mechanisms of telomere maintenance. These results suggest that this strategy could lead to the development of a general approach for the treatment of all human cancers.

15. SUBJECT TERMS
telomere, telomerase, apoptosis, inhibition, mutants, anti-cancer drugs

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Introduction

Breast cancer is one of the most common malignancies among women. Typically after surgical removal of the tumor mass, breast cancer patients are treated with chemotherapeutic drugs that are quite toxic, lack selectivity and often result in resistance. Estrogen-responsive (ER) tumors that initially respond to tamoxifen often develop resistance to this agent and progress to metastatic disease. The new generations of aromatase inhibitors, which have been shown to be highly effective in early breast cancer settings, also eventually induce resistance [1]. Therefore more effective treatments are needed to treat breast cancer.

Telomeres are nucleoprotein structures that cap and protect the end of eukaryotic chromosomes from being recognized as DNA breaks by the DNA repair machinery (reviewed in [2]). Telomeres also counteract the loss of DNA sequences during DNA replication. This phenomenon called the "end replication problem" is due to the inability of conventional DNA polymerases to replicate the ends of linear chromosomes and causes telomere shortening at each cell division. When telomeres become critically short, cells stop dividing and enter a permanent growth arrest called replicative senescence [2]. Since all replicating cells are subject to telomere shortening, cells with indefinite proliferative capacity, such as germ line and stem cells must activate some mechanisms to maintain telomere length. Human cells have at least two mechanisms for telomere maintenance. The most common one is the activation of telomerase, an RNA-dependent DNA polymerase that adds *de novo* telomeric repeats onto the telomeres [3]. The second one called alternative lengthening of telomeres or ALT is used much more rarely than telomerase and relies on homologous recombination and copy switching [4-6]. The telomerase enzyme minimally contains a catalytic subunit with reverse transcriptase activity, called hTERT in humans, and an RNA subunit called hTR with a domain that acts as template for telomere synthesis [3]. In humans telomerase is present in all cells during embryonic development, and in the adult only in germ line, stem cells and at very low level in activated lymphocytes and some proliferating somatic cells [7, 8]. In contrast, telomerase is up-regulated in over 85% of human tumors, indicating that reactivation of the enzyme is a critical step for tumor growth and progression [9, 10]. Therefore telomerase represents a promising target for the development of anti-cancer therapies. Telomerase inhibition in telomerase-positive tumor cells results in erosion of telomeric DNA and ultimately cell death due to dysfunctional telomeres. These effects would be relatively specific to cancer cells with minor side effects for somatic cells and stem cells, which have longer telomeres and slower duplication time. The major limitation of such approach is the time necessary for the telomeres to shorten enough to engage a proliferative arrest. Many reports have shown, in fact, that inhibition of telomerase resulted effectively in death of tumor cells with short telomeres, but not of cells with long telomeres [11-13]. A possible strategy to circumvent this lag phase is to target the telomeres themselves, by causing their uncapping independently of shortening. This approach is based on reprogramming the telomerase enzyme by using hTR variants with mutations in the template region. Reconstitution of a mutant holoenzyme by expressing a mutant hTR in telomerase-positive cells resulted in synthesis of mutant telomeres, decreased cell proliferation and increased apoptosis [14, 15]. These effects were rapid and did not require telomere shortening, suggesting that they were likely caused by alteration of the telomere structure. However, unless the mutant hTR was highly over-expressed or the wild-type hTR (wt-hTR) was not present [16, 17], the effects were mild, probably because telomere function could be restored by addition of wt sequences to mutant ones [14].

Here we propose a new anti-cancer approach based on the combination of telomere disturbances induced by mutant template hTRs and chemotherapeutic drugs. The novelty of this strategy is represented by the fact that telomere disturbances are achieved in a telomerase-dependent manner, thereby maintaining tumor specificity, but without the lag phase associated with all telomerase-based therapies. Moreover it could be applied to all tumors independently of their initial telomere lengths and mechanisms of telomere maintenance.

Body of work

The main aim of this project was to investigate whether interfering with telomere maintenance results in increased sensitivity to anti-cancer agents commonly used to treat breast cancers, such as doxorubicin and paclitaxel, and whether it is possible to develop a therapeutic approach applicable to all cancers independently of their initial telomere lengths and mechanisms used for telomere maintenance. For this purpose we compared the effects of drug treatment in cells in which we interfered with either telomere structure through the introduction of a mutant template hTR, or telomere length maintenance through telomerase inhibition by a dominant-negative hTERT (dn-hTERT).

During the first two years of this project we selected three telomerase-positive breast cancer cell lines with different p53 status and different telomere lengths (TRF) (YCC-B1: short TRF; MCF-7: intermediate TRF; YCC-B2: long TRF; [18]), established stable clonal populations expressing either dn-hTERT or a mutant template hTR (MuA-hTR) [14, 16], and started to analyze their response to commonly used anti-cancer drugs (etoposide, doxorubicin and paclitaxel) through colony forming assays. In this last year we completed our analysis of the cell populations that we have generated and their response to anti-cancer drug treatment and tried to identify the mechanism by which the mutant template hTR increases cell sensitivity to the drugs. All our data have been submitted for publications and are now in press (see appendices [19, 20]).

dn-hTERT cells: In the previous two years we had characterized the clonal populations derived from MCF-7 and YCC-B2 cells expressing the dn-hTERT for their response to both doxorubicin and etoposide and found that in all cases the dn-hTERT-expressing cells were more sensitive to both drugs than the control cells and this effect required overall telomere shortening.

To complete our analysis, we have also treated the dn-hTERT and the vector cells with paclitaxel, a drug largely used for breast cancer chemotherapy that belongs to the group of the taxanes and exerts its anti-tumor activity by stabilizing the microtubules and not by causing DNA damage (see appended paper [19]). We found that colony forming assays of cells expressing dn-hTERT treated with low doses of paclitaxel yielded fewer colonies compared to the vector clone, indicating that telomerase inhibition sensitize cells to drugs with different mechanism of action (see Fig. 1A). However we observed this effect only in the YCC-B2 derivatives. This result is not unexpected, because as shown several times in the literature, sensitivity to drug is often cell line and drug-type dependent. Finally there are several data indicating that estrogen receptor-positive tumors can be treated with well tolerated therapies, whereas estrogen-negative tumors are in greater need of better treatments. MCF-7 cells express the ERα, whereas the ER status of YCC-B2 cells had never been reported. We analyzed by western blot the ER status in YCC-B2 cells and found that these cells don't express the estrogen receptor (see Fig. 1B). Therefore our data show that telomerase inhibition could be used as a general approach to induce cell death in all breast tumors regardless of their estrogen receptor status. In all cases the anti-proliferative effects of telomerase inhibition required prior telomere shortening.

MuA-hTR cells: We also completed our analysis of the cell populations expressing the mutant template hTR. We had previously found that the introduction of a mutant hTR in telomerase-positive breast cancer cell lines caused telomere disturbances that enhanced their sensitivity to both DNA-damaging agents, such as doxorubicin and etoposide, and to microtubule-stabilizing drugs, such as paclitaxel. We also showed that these effects were present immediately after the isolation of the clonal derivatives, independently of the initial telomere lengths and did not require overall telomere shortening.

The main goal of our approach was to target telomere structure rather than length to overcome the lag phase associated with telomerase inhibition, which has been one of the main limitations for the clinical applicability of telomerase-based strategies.

The use of stable clonal populations, whose establishment requires a certain number of cell divisions (around 20 population doublings (PD)), did not allow us to exclude the presence of a lag

phase before the mutant hTR-dependent effects were induced. To confirm that no lag phase was required, we used a polyclonal population expressing the mutant hTR that could be established and analyzed within a limited number of cell divisions. We found that the presence of the mutant template hTR for only few cell divisions in YCC-B2 cells was sufficient to increase their sensitivity to doxorubicin treatment (see Fig. 2A). This result is in agreement with our previous observation that the mutant hTR does not induce any change in telomere length in the clonal populations analyzed. We also used Q-FISH (quantitative fluorescence in situ hybridization) to analyze at the single cell level whether the introduction of mutant repeats into the telomeres affected the telomere structure and stability. We found an increase in telomere length heterogeneity in the clones expressing MuA-hTR compared to the control cells (Fig. 3A). However, we did not observe any significant increase in the number of signal free ends or in the frequency of chromosome fusions (data not shown). Finally the lengths of the G-tails were not affected in cells expressing the mutant RNA (Fig. 3B), indicating that the single-stranded 3'-overhangs were properly maintained. These results are in agreement with our observation that the presence of the mutant hTR by itself had only a mild effect on viability and proliferative ability both in mass culture and in colony forming assays (Fig. 4), most likely because telomere disturbances induced by the mutant RNA could be counterbalanced by the addition of wt repeats by the endogenous telomerase.

We and others had previously shown that the mutant hTR-dependent effects required the presence of a biologically active telomerase enzyme [16]. As a control for our experiments we used ALT VA13 cells that do not express either hTERT or hTR, transfected them with MuA-hTR, selected clonal populations and treated them with doxorubicin (Fig. 2B). In contrast with the results in telomerase-positive cells, the introduction of the mutant hTR in ALT VA13 cells did not significantly affect the response to doxorubicin treatment, demonstrating that the effects induced by the mutant template hTR are dependent on the presence of an active telomerase enzyme (Fig. 2C).

In this last year we also tried to identify the mechanism by which the mutant template hTR increases cell sensitivity to anti-cancer drugs. For this purpose we compared the cell cycle profile in the vector and the mutant hTR clones derived from the YCC-B2 cells with and without doxorubicin treatment. In the absence of drug, the cell cycle profiles of the mutant hTR clones were comparable to the control cells. After 24h of doxorubicin treatment, vector cells showed an alteration of the cell cycle profile with a marked decrease in the percentage of cells in G1, a slight increase in G2/M and in the subG1 fractions compared to untreated cells (Fig. 5A). These characteristics are indicative of mitotic catastrophe, a process of cell death that occurs in response to several anti-cancer agents [21, 22]. Interestingly in the clonal populations expressing the mutant hTR the effects of doxorubicin treatment were more pronounced than in the vector cells: the percentage of cells in G1 was further decreased compared to the controls whereas the percentage of cells in G2/M was similar (Fig. 5A). After 72h, the main effect of doxorubicin treatment was a marked increase in the percentage of cells in subG1, which was significantly more pronounced in the mutant clones than in the vector (Fig. 5B). Thus, the reduction in the number of colonies following drug treatment in the mutant populations could be attributed to an exacerbation of the anti-proliferative effects of doxorubicin in the presence of the mutant hTR, resulting in an alteration of the cell cycle profile associated with high levels of cell death.

A previous study has shown that expression of high levels of mutant template hTRs induces a DNA damage response with the formation of nuclear foci at the telomeres that co-localize with the DNA-damage protein 53BP1 [23]. In order to understand whether that was the case also in our populations which express low levels of the mutant hTR, we monitored the presence of 53BP1 foci in vector and MuA-hTR YCC-B2 clones. We found that the mutant populations had a significantly higher percentage of cells containing 53BP1 foci compared to the vector cells, although only few foci/cell were detected (1-4 foci/cell; Fig. 5C). Doxorubicin treatment caused an increase of both the percentage of foci-containing cells and the number of foci/cell in all populations analyzed, whereas treatment with paclitaxel, which does not induce primarily DNA damage, did not affect these parameters. These

results suggest that the presence of the mutant template hTR causes a slight disturbance of the telomere structure which results in the formation of DNA damage foci; given the low level of MuA-hTR expression and the presence of the wild-type enzyme, these effects are mild and do not affect cell viability. When cells are treated with anti-cancer drugs, however, these disturbances of the telomere cap exacerbate the anti-proliferative effects of the drugs, causing high levels of cell death.

Key research accomplishments

- Telomerase inhibition by dn-hTERT sensitizes breast cancer cells to anti-cancer drugs with different mechanisms of actions (i.e. doxorubicin, etoposide, paclitaxel)
- dn-hTERT sensitizes to anti-cancer drugs both ER-positive and ER-negative breast cancer cells
- The presence of the MuA-hTR does not affect the telomere profile nor the length of the G-tails
- The mutant hTR-dependent effects require a biologically active telomerase enzyme
- Identification of a possible mechanism by which the MuA-hTR increases cell sensitivity to anti-cancer drugs

Reportable outcomes

- Meeting presentation: Cerone MA, Autexier C, Londoño-Vallejo JA and Bacchetti S "A human cell line that maintains telomeres in the absence of telomerase and of key markers of ALT" EMBO Workshop on "Chromosome structural elements: from DNA sequence to function". Villa Mondragone, Rome, Italy; 29 September - 3 October, 2005
- Manuscript: Cerone MA, Londoño-Vallejo JA and Autexier C (2006) "Telomerase inhibition enhances the response to anti-cancer drug treatment in human breast cancer cells" *Molecular Cancer Therapeutics* (in press)
- Manuscript: Cerone MA, Londoño-Vallejo JA and Autexier C (2006) "Mutated telomeres sensitize tumor cells to anticancer drugs independently of telomere shortening and mechanisms of telomere maintenance" *Oncogene* (Epub)
- Manuscript: Marie-Egyptienne D, Cerone MA, Londoño-Vallejo JA and Autexier C. (2005) "A human-Tetrahymena pseudoknot chimeric telomerase RNA reconstitutes a non-processive enzyme in vitro that is defective in telomere elongation" *Nucleic Acids Res,* 33(17):5446-57

Conclusions

Telomere maintenance is an essential requisite for indefinite cell proliferation and for tumor progression. Therefore, interfering with this process in cancer cells may be used as a therapeutic approach to block the growth of tumor cells. Recent data in the literature and our data in breast cancer cell lines have shown that inhibition of telomerase per se or in combination with anti-cancer drugs can induce cell death but requires prior telomere shortening. Similarly targeting the RNA subunit of telomerase by introducing mutant template RNAs resulted in telomere destabilization and loss of cell viability, although the effects were not dramatic due to the presence of the wild-type hTR.

The aim of this study was to investigate the feasibility of a new approach based on the combination of telomere destabilization induced by mutant template hTRs and chemotherapeutic drugs to induce rapid tumor cell death. Our results show that interfering with telomere structure maintenance in breast cancer cells dramatically increases the susceptibility of these cells to commonly used anti-

cancer drugs with different mechanisms of action (i. e. etoposide, doxorubicin and paclitaxel). More interestingly, these effects are independent of the initial length of the telomeres and do not require telomere shortening, eliminating the lag phase associated with telomerase-based strategies. We also showed that reconstitution of a mutant telomerase enzyme in telomerase-negative ALT cells can be used as a way to sensitize telomerase-independent immortal cells to chemotherapeutics, without any effect on telomere length maintenance nor cell proliferation.

In order to understand the mechanism by which the mutant template hTR increases the susceptibility of cancer cells to chemotherapeutics, we analyzed the cell cycle profile and monitored the presence of DNA-damage foci in the vector and the mutant populations. We found a higher percentage of cells containing 53BP1 foci in the mutant clones compared to the vector cells, indicating that the introduction of mutated repeats causes a slight disturbance of the telomere structure and the activation of a DNA damage response. In normal culture condition given the low level of MuA-hTR expression and the presence of the wild-type enzyme, these effects are mild and do not affect cell viability. When cells are treated with anti-cancer drugs, however, these disturbances of the telomere cap exacerbate the anti-proliferative effects of the drugs. Indeed cell cycle analyses after doxorubicin treatment showed a marked alteration of the cell cycle profile with high levels of cell death and these effects are more pronounced in the mutant clones compared to the controls, resulting in higher sensitivity to drug treatment.

As for all telomerase-based therapies, the mutant hTR-based approach would affect all telomerase-positive cells, including stem cells and some somatic cells that express low levels of telomerase. However the low levels of telomerase and the longer duplication times of these cell populations imply that the side effects of telomere disturbances on normal cells should be minor. Moreover stem cells proliferate only in response to specific stimuli to maintain tissue turnover; this creates a possible therapeutic window that could be exploited.

Altogether, our data indicate that interfering with telomere structure in cancer cells through the introduction of mutant template hTR may represent an effective and general strategy to block tumor cell growth. More importantly this strategy may lead to the development of a clinical approach for the treatment of all tumors independently of their initial telomere lengths and mechanisms to maintain them and would allow the use of lower levels of chemotherapeutics or shorter treatment times thereby reducing systemic cytotoxicity.

Training accomplishments

During my last year of this project I have further improved my skills in culture of human cells as well as biochemistry and molecular biology. I also have continued fruitful scientific collaborations with researchers (see papers) and graduate students in this institute and outside this institute for both experimental supports as well as technical and scientific discussions. Finally I have tutored several younger students in the lab both for experimental work and for scientific suggestions on their projects.

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Figure 1

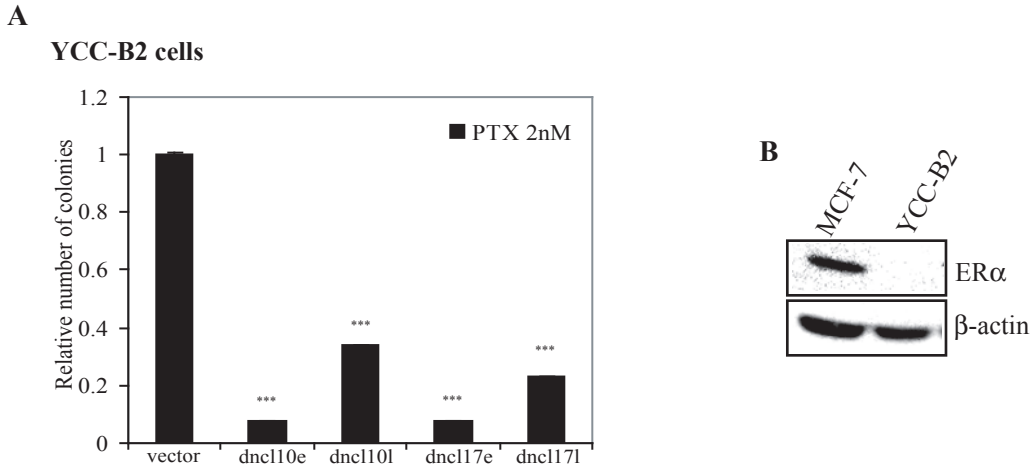

Figure 1. (A) Introduction of dn-hTERT increases the sensitivity of breast cancer cells to paclitaxel. Colony forming assay of YCC-B2 vector and dn-hTERT clones treated with the indicated concentrations of paclitaxel (PTX). The relative numbers of colonies were obtained as a ratio of the colonies in the dn-hTERT clones over the colonies in the vector clones. Values ± SEM of at least three independent experiments are shown. Statistical differences between the vector and each dn-hTERT clone were calculated with the unpaired t test, using the online GraphPad QuickCalcs software. e, early PDs < 25; l, late PDs >40. *=p<0.05; **=p<0.01; ***=p<0.001. **(B)** Analysis of ER expression in the breast cancer cell lines used in the study. The ER status of YCC-B2 and MCF-7 cells has been analyzed by western blot using 50 µg of cellular extracts. β-actin was used as a loading control.

Figure 2

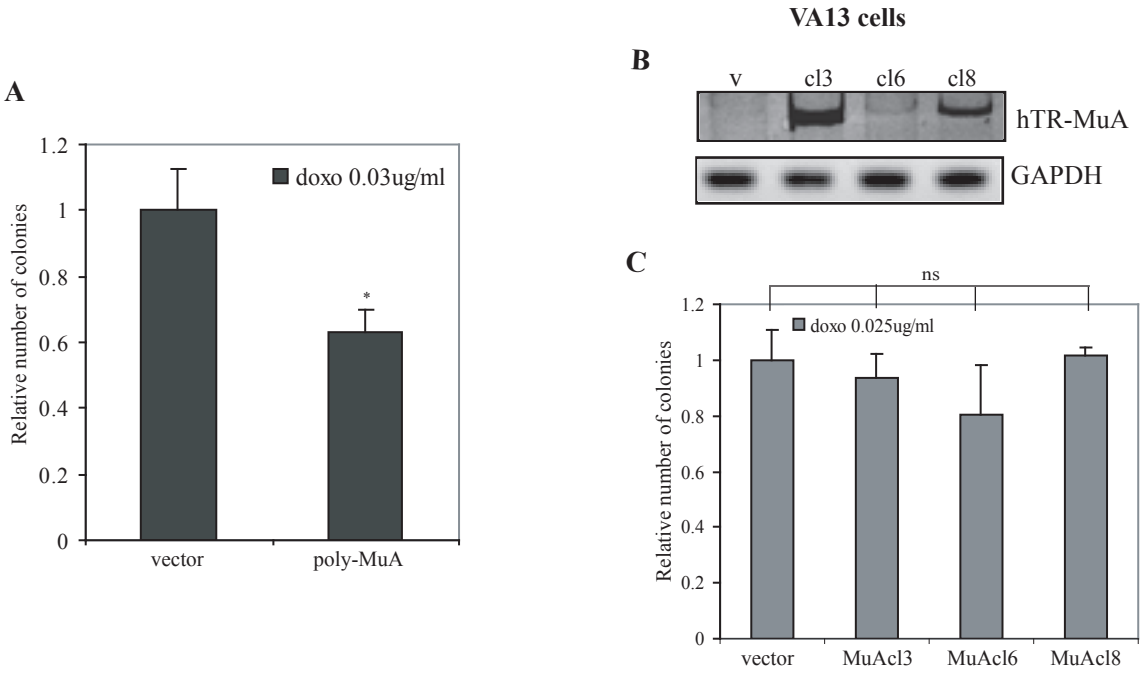

Figure 2. (A) Colony forming assay of the vector and the polyclonal population expressing the mutant hTR derived from the YCC-B2 cells treated with the indicated concentration of doxorubicin (doxo). **(B-C)** Increased drug sensitivity due to the mutant hTR requires active telomerase. (B) The expression of MuA-hTR was analyzed by RT-PCR in one vector- and three mutant clones from VA13 cells. GAPDH was used as a control. (C) Colony forming assay of the VA13 clones as in (B) treated with the indicated concentration of doxorubicin. The relative numbers of colonies were obtained as a ratio of the colonies in the mutant clones over the colonies in the vector clone. Values ± SEM of at least three independent experiments are shown. *p<0.05; ns, not significant.

Figure 3

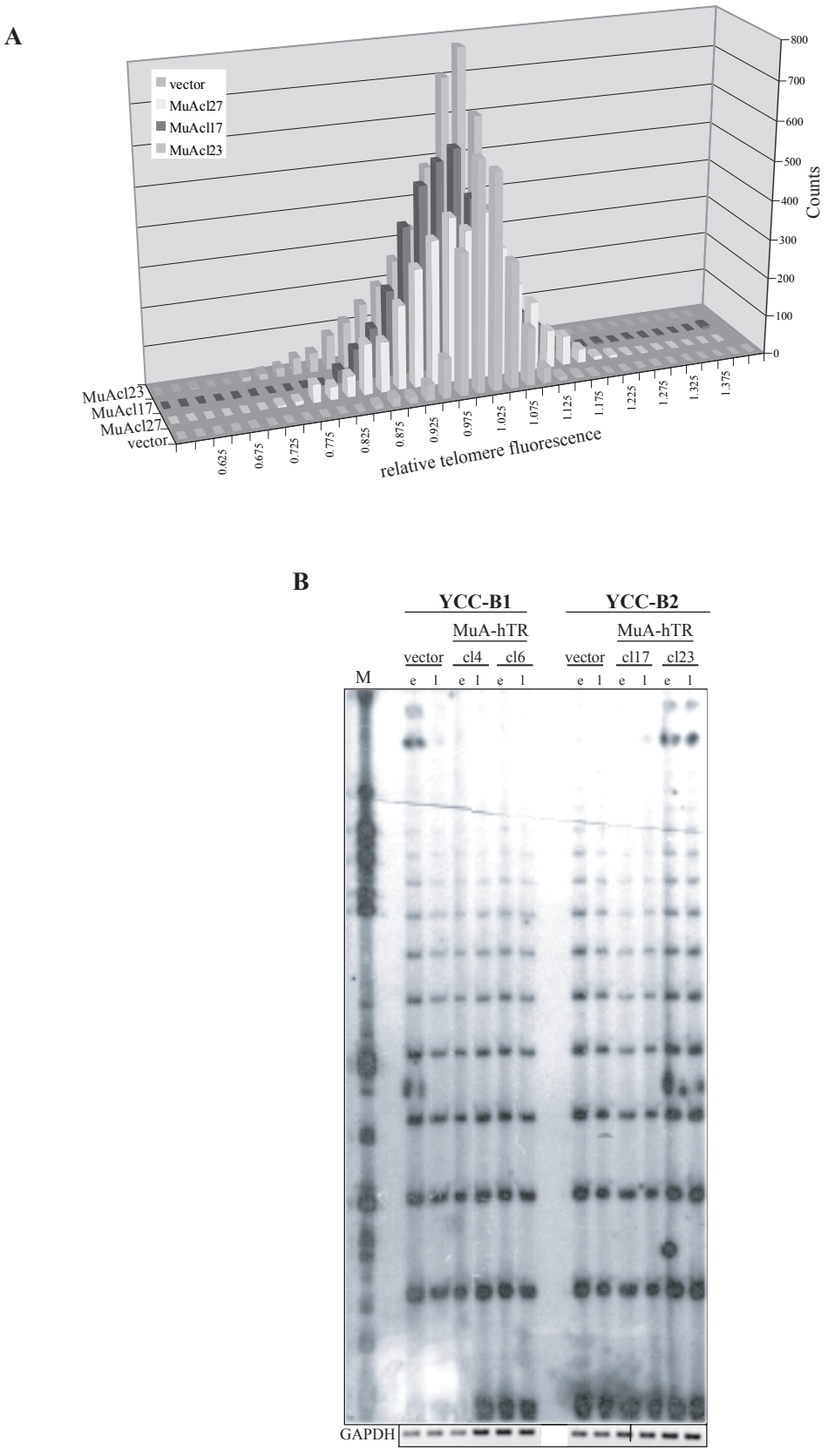

Figure 3. (A) The telomere length profile of YCC-B2 clones expressing the vector or MuA-hTR was analyzed by Q-FISH and the distributions of relative telomere fluorescence intensities are shown. Although overall telomere length is maintained, there is a broader distribution of relative telomere lengths within cells carrying the mutant RNA. **(B)** The lengths of the G-tails was analyzed with the T- assay (telomere-oligonucleotide ligation assay) using 5µg of genomic DNA from YCC-B1 and YCC-B2 derivatives at early (e) and late (l) PDs. GAPDH was used as a loading control.

Figure 4

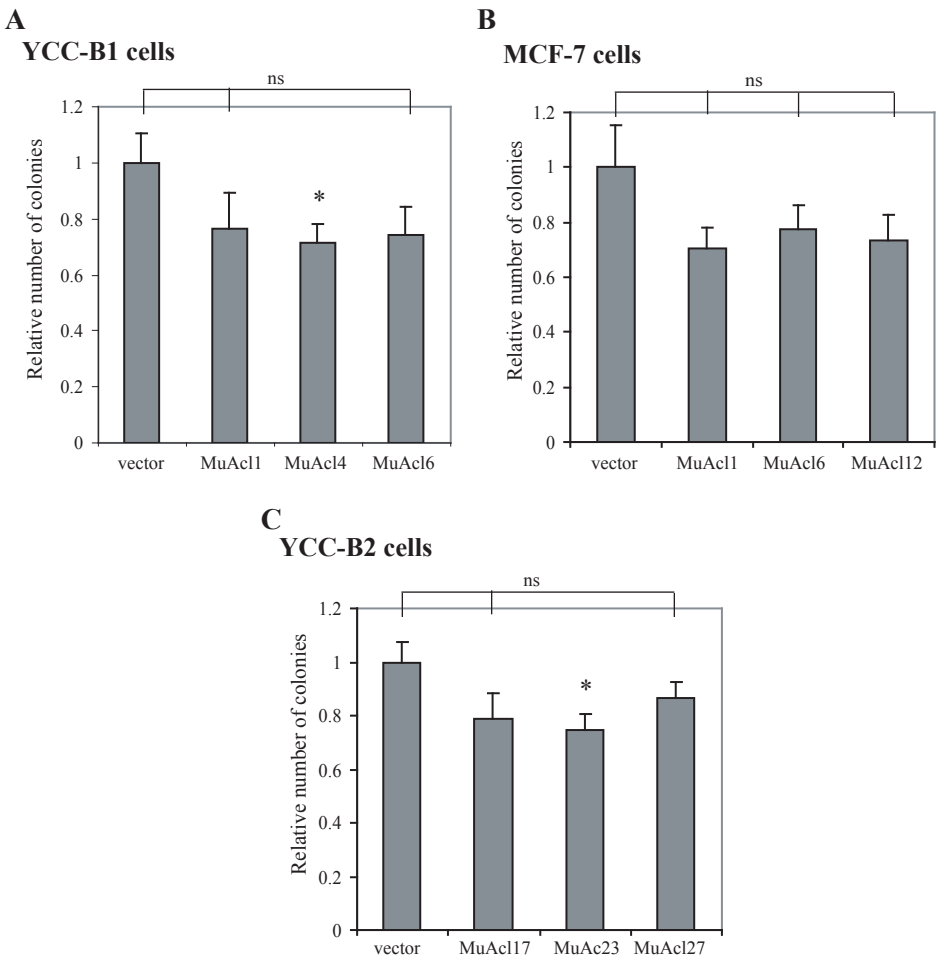

Figure 4. Effect of the MuA-hTR on cell viability and proliferative ability. (A-C) Colony forming assays of one vector and three MuA-hTR clones from YCC-B1 (A), MCF-7 (B) and YCC-B2 (C) cells in the absence of drug treatment. The relative numbers of colonies were obtained as a ratio of the colonies in the mutant clones over the colonies in the vector clones. Values ± SEM of at least four independent experiments are shown. Statistical differences between the vector and each mutant clone were calculated with the unpaired t test. *$p<0.05$; ns, not significant.

Figure 5

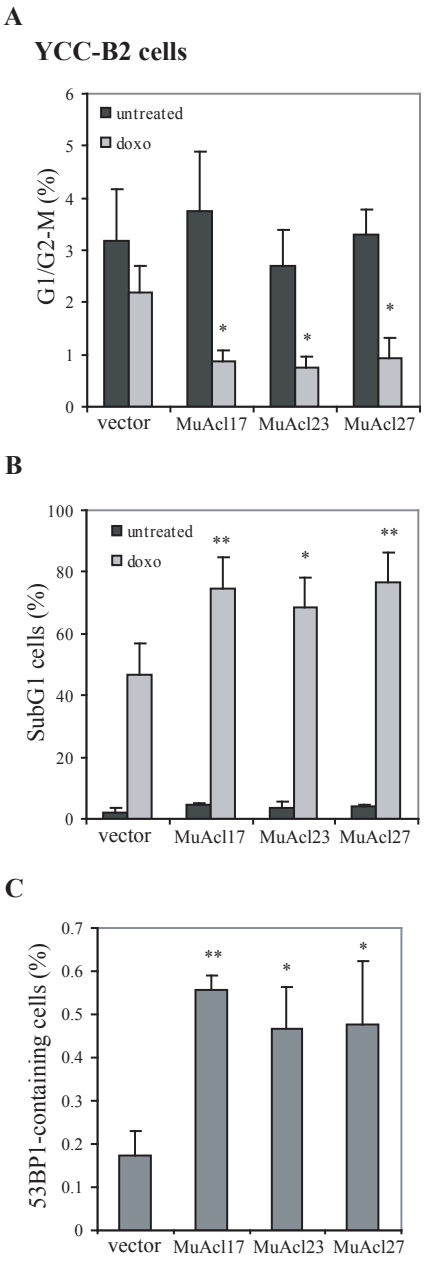

Figure 5. Doxorubicin treatment alters the cell cycle profile in MuA-hTR-expressing YCC-B2 cells. **(A)** The cell cycle profile of one vector and three mutant hTR clones was analyzed one day after doxorubicin treatment and the values of the ratio between the percentage of cells in G1 and in G2-M are indicated. As a comparison, the G1/G2-M ratios in the untreated cells are shown. **(B)** The percentage of subG1 cells was analyzed in vector and mutant hTR clones either left untreated or treated for 3 days with doxorubicin. **(C)** MuA-hTR induces the formation of 53BP1 foci. The percentage of cells containing 53BP1 foci in the vector and mutant hTR YCC-B2 clones is shown. Values represent the means ± s.d. of at least three independent experiments. Statistical differences between the vector and each mutant clone were calculated with the unpaired t test and are indicated as *$p<0.05$ and **$p<0.01$.

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ORIGINAL ARTICLE

Mutated telomeres sensitize tumor cells to anticancer drugs independently of telomere shortening and mechanisms of telomere maintenance

MA Cerone[1,2], JA Londoño-Vallejo[3] and C Autexier[1,2]

[1]Department of Anatomy and Cell Biology, McGill University, Quebec, Canada; [2]Bloomfield Centre for Research in Aging, Lady Davis Institute, Sir Mortimer B. Davis Jewish General Hospital, Quebec, Canada; and [3]Telomeres and Cancer Laboratory, CNRS-IC-UPMC, Institut Curie, Paris, France

Telomerase is a ribonucleoprotein complex that maintains the stability of chromosome ends and regulates replicative potential. Telomerase is upregulated in over 85% of human tumors, but not in adjacent normal tissues and represents a promising target for anticancer therapy. Most telomerase-based therapies rely on the inhibition of telomerase activity and require extensive telomere shortening before inducing any antiproliferative effect. Disturbances of telomere structure rather than length may be more effective in inducing cell death. Telomerase RNA subunits (hTRs) with mutations in the template region reconstitute active holoenzymes that incorporate mutated telomeric sequences. Here, we analysed the feasibility of an anticancer approach based on the combination of telomere destabilization and conventional chemotherapeutic drugs. We show that a mutant template hTR dictates the synthesis of mutated telomeric repeats in telomerase-positive cancer cells, without significantly affecting their viability and proliferative ability. Nevertheless, the mutant hTR increased sensitivity to anticancer drugs in cells with different initial telomere lengths and mechanisms of telomere maintenance and without requiring overall telomere shortening. This report is the first to show that interfering with telomere structure maintenance in a telomerase-dependent manner may be used to increase the susceptibility of tumor cells to anticancer drugs and may lead to the development of a general therapy for the treatment of human cancers.
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Correspondence: Dr C Autexier, Department of Anatomy and Cell Biology, Lady Davis Institute, Sir Mortimer B. Davis Jewish General Hospital, McGill University, 3755 Cote Ste Catherine, Montreal H3T 1E2 Quebec, Canada.
E-mail: chantal.autexier@mcgill.ca
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Introduction

Telomerase is a ribonucleoprotein essential for the maintenance of the structure and the length of telomeres, the nucleoprotein complexes at the ends of eucaryotic chromosomes that cap and protect them from degradation, fusions and recombination. Telomerase contains two essential subunits, a catalytic component with reverse transcriptase activity, called human telomerase reverse transcriptase (hTERT) in humans, and an RNA moiety, called hTR, which functions as a template for the synthesis of T_2AG_3 repeats onto the chromosome termini. In humans, telomerase is expressed during embryonic development, but is repressed in adult tissues, with the exception of germ line and stem cells, which remain positive for the enzyme and maintain telomere length (Collins and Mitchell, 2002). Some proliferating somatic cells also have low levels of telomerase activity sufficient for the maintenance of telomere structure but not length (Masutomi *et al*., 2003). In contrast, telomerase is detected in most immortalized cell lines and in over 85% of human cancers, indicating that its reactivation is an essential step for unlimited proliferation and cancer progression (Shay and Bacchetti, 1997). Telomerase may therefore represent an excellent anticancer target. Anti-telomerase approaches rely on the loss of telomerase activity and result in erosion of telomeric DNA, which causes genomic instability and cell death. These effects are predicted to be relatively specific to cancer cells with minor side effects for somatic cells and stem cells, which have longer telomeres, no or low levels of telomerase and slower duplication time. The major limitation of telomerase inhibition is the time necessary for the telomeres to become critically short before the antiproliferative effects are observed. Several reports have shown, in fact, that inhibition of telomerase by either a dominant-negative protein or antisense oligonucleotides against hTR results effectively in death of tumor cells with short but not long telomeres (Hahn *et al*., 1999; Herbert *et al*., 1999; Zhang *et al*., 1999). A complementary strategy to block tumor cell growth is to use telomerase inhibition as a means to sensitize cancer cells to chemotherapeutic drugs or angiogenesis inhibitors (Kondo *et al*., 1998; Ludwig *et al*., 2001; Misawa *et al*., 2002; Chen *et al*., 2003; Tentori *et al*.,

2003). However, the responsiveness to such treatments appears to vary in a cell- and drug-type-dependent fashion (Folini *et al*., 2000; Tentori *et al*., 2003) and in most cases requires prior telomere shortening (Kondo *et al*., 1998; Misawa *et al*., 2002; Chen *et al*., 2003; Ward and Autexier, 2005), indicating that even combination approaches based on telomerase inhibition may not be clinically suitable for cancer treatment.

Telomere uncapping, independent of shortening, represents a way to eliminate the lag phase associated with telomerase inhibition. Expression of hTRs with mutations in the template sequences results in the synthesis of mutated repeats that cause telomere structure disturbances and reduced cell viability. These effects are most likely owing to the inability of telomeric proteins to bind to the mutated repeats and do not require telomere shortening (Marusic *et al*., 1997; Kim *et al*., 2001). However, unless the mutant hTR is highly overexpressed or the endogenous hTR is absent (Guiducci *et al*., 2001; Li *et al*., 2004), the endogenous telomerase complex adds wild-type sequences to mutant ones, partially restoring telomere function and mitigating the deleterious effects of the mutant telomerase. Hence, the expression of mutant template RNAs may only be partially effective in inducing cell death of telomerase-positive cancer cells and human tumors.

The goal of the current work is to validate a different approach for anticancer treatment based on the combination of telomere destabilization induced by the introduction of mutant template hTRs and anticancer drugs. The advantage of this strategy is that telomere disturbances are achieved in a telomerase-dependent manner, thereby maintaining tumor selectivity, but without the lag phase associated with telomerase-based therapies. We found that telomere destabilization induced by the introduction of mutant hTRs increased the susceptibility of human breast cancer cells to chemotherapeutic drugs independently of their initial telomere length and without requiring bulk telomere shortening. Moreover, we showed that ALT (alternative lengthening of telomeres) GM847 cells, engineered to express both hTERT and the mutant hTR, became more sensitive to drug treatments compared to controls. These data indicate that interfering with the maintenance of telomere structure in combination with anticancer drugs may be exploitable as a general anticancer approach to target cancer cells independently of telomere length and mechanisms of telomere maintenance.

Results

Establishment of stable clonal populations expressing a mutant template hTR

We investigated whether telomere destabilization induced by mutant template hTRs could increase the susceptibility of tumor cells to anticancer drugs without the lag phase associated with telomerase inhibition. For this purpose, we screened several human breast cancer cell lines and chose three telomerase-positive cell lines with different telomere lengths and p53 status (Figure 1): YCC-B1 cells with short telomeres (average terminal restriction fragment (TRF) 3.2 kb), MCF-7 cells with intermediate telomere length (average TRF 7 kb) and YCC-B2 cells with long telomeres (average TRF 11 kb). YCC-B1 and MCF-7 cells had wild-type p53, which was induced upon doxorubicin treatment, a known p53 activator; YCC-B2 cells had undetectable levels of p53, which was not induced upon doxorubicin treatment and did not induce p21 expression (Figure 1c and data not shown).

We disturbed telomere maintenance in these cells through the expression of an hTR with a point mutation in the template region (MuA-hTR). This RNA reconstitutes a mutant holoenzyme that adds mutant repeats (T_3G_3) onto the telomeres (Marusic *et al*., 1997; Guiducci *et al*., 2001; Kim *et al*., 2001). YCC-B1, MCF-7 and YCC-B2 cells were transfected with the vector alone or a plasmid encoding MuA-hTR and clonal populations were selected. For each cell line, one vector clone and three clonal populations expressing the mutant RNA were selected for further studies (Figure 2 and Table 1). The presence of the mutant hTR was monitored by telomere repeat amplification protocol

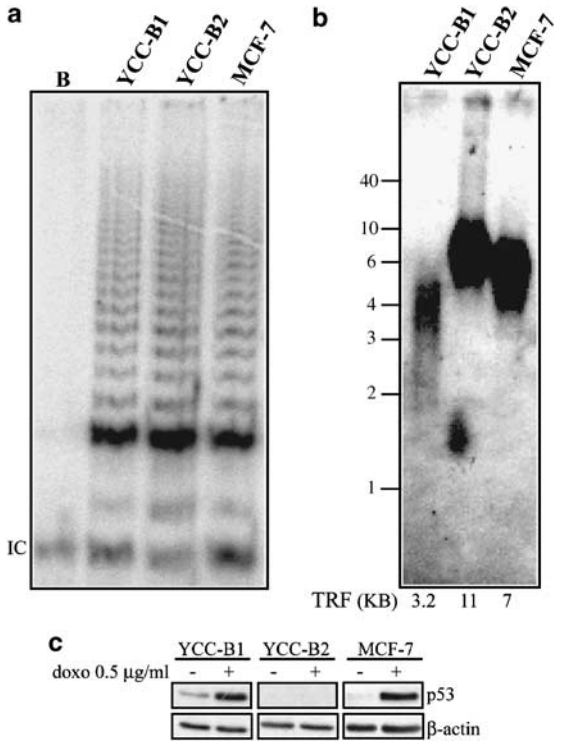

Figure 1 Characterization of the human breast cancer cell lines chosen for the study. (**a**) Telomerase activity was analysed by TRAP assay using 0.5 μg of whole-cell extracts. IC indicates the internal PCR control. B indicates the blank. (**b**) TRF lengths of YCC-B1, YCC-B2 and MCF-7 cells were analysed by PFGE. The average telomere length calculated by densitometric scanning of each lane is shown at the bottom. Representative molecular weight markers are indicated. (**c**) The p53 status has been analysed by Western blot using 40 μg of cellular extracts from YCC-B1, YCC-B2 and MCF-7 cells. A 0.5 μg/ml portion of doxorubicin (doxo) was used as a standard concentration for p53 induction. β-Actin was used as a loading control.

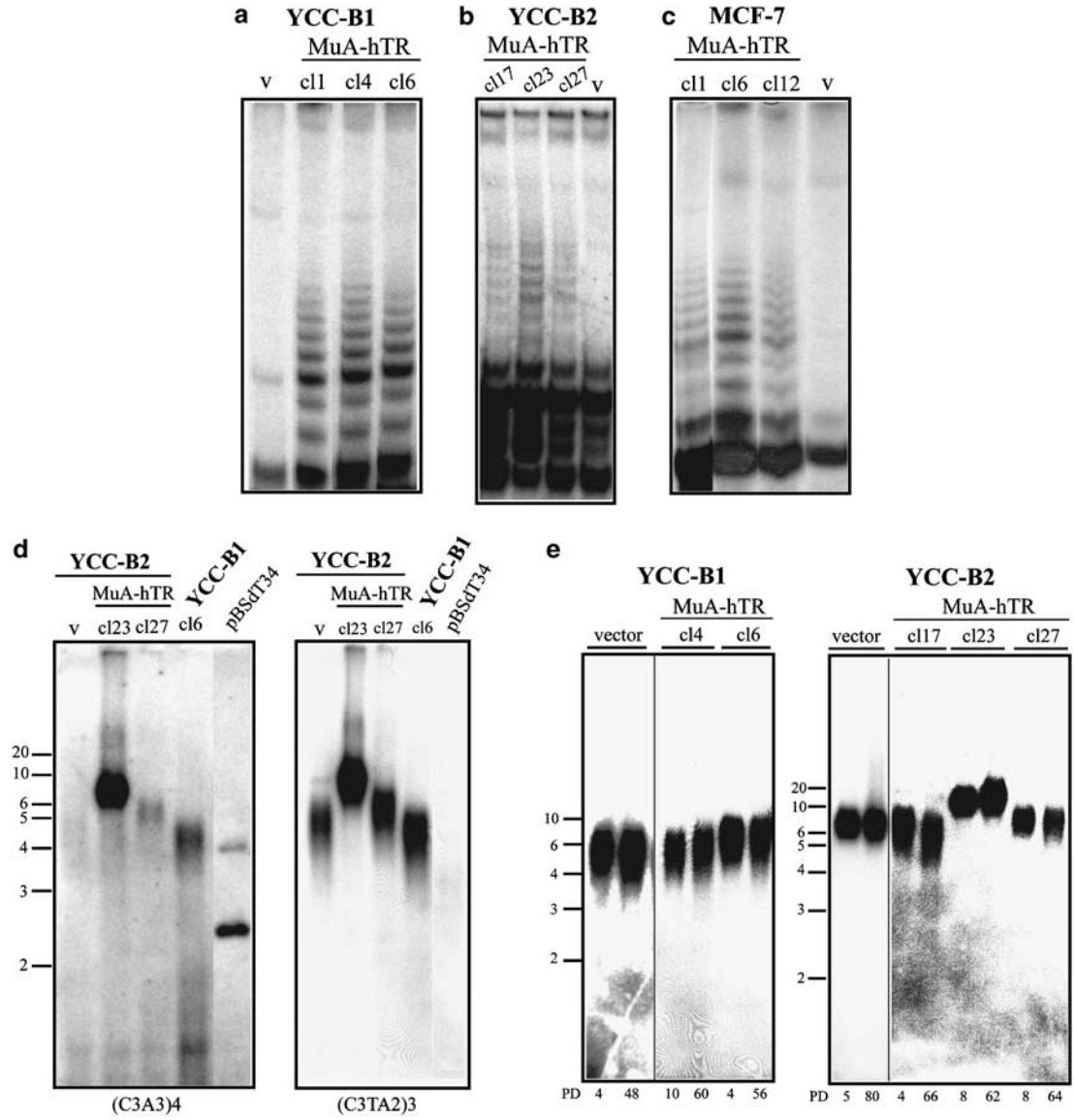

Figure 2 (**a**–**c**). MuA-hTR reconstitutes a catalytically active telomerase enzyme. Cell extracts (5–7 μg) from vector and MuA-hTR YCC-B1 (**a**), YCC-B2 (**b**) and MCF-7 (**c**) clones (cl) were assayed for the telomerase activity specified by the mutant hTR. (**d**) Mutated repeats specified by MuA-hTR are incorporated into the telomeres. TRF analysis of vector- and MuA-hTR-expressing clones with a MuA-specific telomeric probe (left panel) and a wild-type probe (right panel). pBSdT34 was used as a positive control for the hybridization with the mutant probe. (**e**) Expression of MuA-hTR does not have any effect on telomere length. TRF lengths in YCC-B1 (left panel) and YCC-B2 (right panel) cells expressing the mutant hTR at early and late PDs were analysed by PFGE. Representative molecular weight markers in kb are indicated.

Table 1 Clonal populations used in the study

Cell line	*MuA-hTR clones*
YCC-B1	MuAcl 1, MuAcl 4, MuAcl 6
MCF-7	MuAcl 1, MuAcl 6, MuAcl 12
YCC-B2	MuAcl 17, MuAcl 23, MuAcl 27

(TRAP) assays under conditions that specifically detect mutant telomerase activity (Figure 2a–c). Wild-type telomerase activity was monitored using standard TRAP conditions and was not affected by the presence of the mutant RNA (data not shown; Feng *et al*., 1995; Marusic *et al*., 1997; Kim *et al*., 2001). The mutant hTR was able to reconstitute an active telomerase that synthesized mutated telomeric repeats (Figure 2d and data not shown). The presence of mutant repeats had only a mild effect on viability and proliferative ability of the mutant hTR clones compared to vector cells both in mass culture and in colony-forming assays, most likely because telomere function could be restored by the addition of wild-type repeats to mutant ones by the endogenous telomerase (Supplementary Figure 1S; Marusic *et al*., 1997). However, as previously reported, cells expressing the mutant RNA formed smaller colonies than the control cells when plated at low

density (data not shown). In addition, no difference in the cell cycle profile of the mutant hTR clones compared to the control cells was observed (Supplementary Figure 1SE and data not shown). The mutant hTR-dependent effects require the presence of a biologically active telomerase enzyme (Guiducci *et al*., 2001). To confirm the dependence of MuA-hTR action on a functional telomerase, we used ALT VA13 cells that do not express hTERT, transfected them with MuA-hTR and selected clonal populations (Supplementary Figure 3SA).

The presence of MuA-hTR increases drug sensitivity without requiring overall telomere shortening

In order to investigate whether telomere destabilization following mutant hTR expression could rapidly sensitize tumor cells to anticancer drugs, we analysed the response of cells expressing the mutant template RNA to two commonly used DNA-damaging agents, etoposide and doxorubicin. The response to the drugs was evaluated by colony-forming assays, which measure the ability of each cell in the population to recover from drug treatment and proliferate. Vector- and MuA-hTR-expressing cells were treated for 24 h with either etoposide or doxorubicin at concentrations ranging between the IC_{15} and IC_{25} of the parental cells and subsequently plated at low density and allowed to proliferate (Figure 3). YCC-B1 cells expressing the mutant hTR showed a significant reduction in the number of colonies compared to the vector-transfected cells upon treatment with either drug, indicating that telomere disturbances induced by the mutant RNA enhanced their sensitivity to the drugs (Figure 3a). Similar results were obtained with the MuA-hTR clones derived from MCF-7 cells and most interestingly with the YCC-B2 derivatives (Figure 3b and c). The observation that cell lines with different telomere lengths responded similarly to drug treatments in the presence of the mutant RNA indicated that the reconstituted mutant holoenzymes affected telomere structure independently of the initial telomere lengths, most likely by disturbing the binding of telomeric proteins and the formation of a proper cap. The mutant hTR-dependent antiproliferative effects were present immediately after the isolation of the clonal derivatives, without any lag phase (Figure 3 and data not shown). TRF analyses showed that telomere length was maintained over time in all clonal populations (Figure 2e and data not shown), indicating that the increased sensitivity to the drugs did not require overall telomere shortening. Quantitative fluorescence *in situ* hybridization (Q-FISH) analyses at the single cell level revealed an increase in telomere length heterogeneity in the clones expressing MuA-hTR compared to the

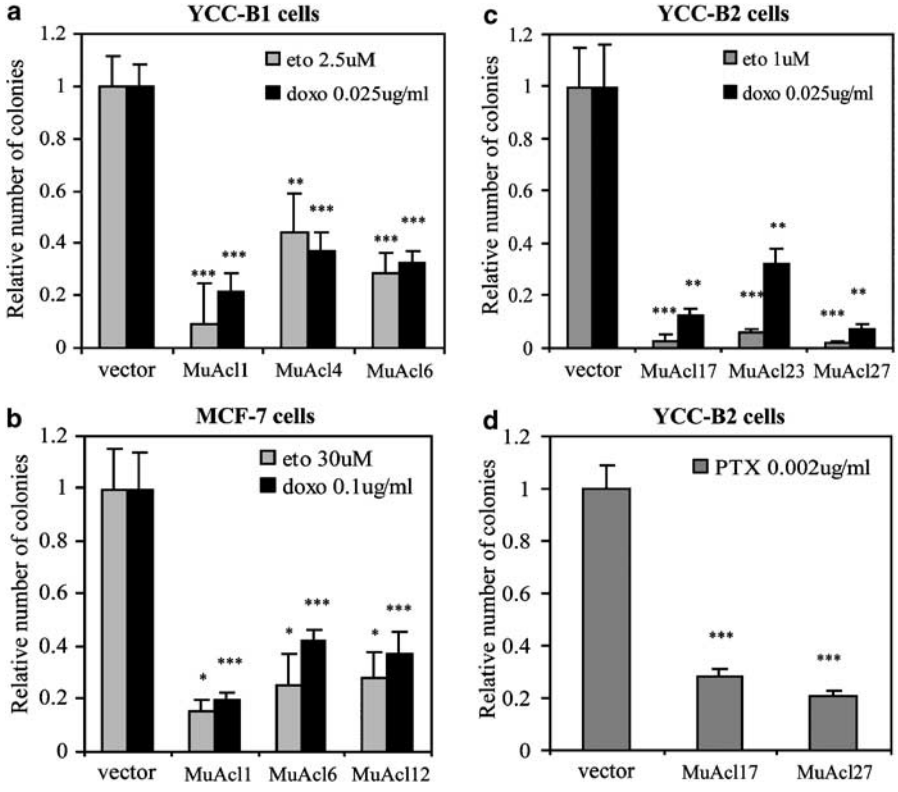

Figure 3 Introduction of MuA-hTR increases the sensitivity of breast cancer cells to anticancer drugs independently of telomere length. (**a–c**) Colony-forming assays of one vector and three MuA-hTR clones from YCC-B1 (**a**), MCF-7 (**b**) and YCC-B2 (**c**) cells treated with doxorubicin (doxo) or etoposide (eto) for 24 h and plated at low density. (**d**) Colony-forming assay of one vector and two MuA-hTR clones from YCC-B2 cells treated with paclitaxel (PTX). The relative numbers of colonies were obtained as a ratio of the colonies in the mutant clones to the colonies in the vector clones. Values ± s.e.m. of at least three independent experiments are shown. Statistical differences between the vector and each mutant clone were calculated with the unpaired *t*-test, using the online GraphPad QuickCalcs software. $*P<0.05$; $**P<0.01$; $***P<0.001$.

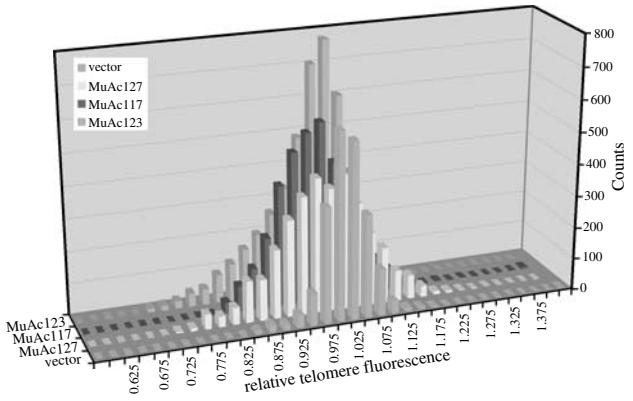

Figure 4 The telomere length profile of YCC-B2 clones expressing the vector or MuA-hTR was analysed by Q-FISH and the distributions of relative telomere fluorescence intensities are shown. Although overall telomere length is maintained, there is a broader distribution of relative telomere lengths within cells carrying the mutant RNA.

control cells (Figure 4 and Supplementary Figure 2S). However, no signal-free ends or significant increase in the frequency of chromosome fusions were observed (Supplementary Figure 2S and data not shown). Finally, the lengths of the G-tails were not affected in cells expressing the mutant RNA (data not shown).

In contrast with the results in telomerase-positive cells, the introduction of the mutant hTR in VA13 cells that do not express hTERT did not have any significant effect on the response to doxorubicin treatment, demonstrating that the effects induced by the mutant template hTR require a functional telomerase enzyme (Supplementary Figure 3SB).

Etoposide and doxorubicin are both DNA-damaging agents. Some studies have suggested that telomerase may play an active role in the DNA damage response in human cells and therefore its inhibition results in increased sensitivity specifically to DNA-damaging compounds (Lee *et al*., 2001; Sharma *et al*., 2003; Shin *et al*., 2004; Masutomi *et al*., 2005; Nakamura *et al*., 2005). To investigate whether the enhanced response to drug treatment in cells expressing MuA-hTR was specific for DNA-damaging drugs, such as etoposide and doxorubicin, we treated MuA-hTR- and vector-transfected YCC-B2 cells with paclitaxel, a commonly used drug that exerts its antitumor activity primarily by stabilizing the microtubules (Jordan *et al*., 1993). We found that MuA-hTR clones were significantly more sensitive to paclitaxel than control cells (Figure 3d). These results indicate that the incorporation of mutated telomeric repeats makes YCC-B2 cells more susceptible to different harmful stimuli, and not specifically to DNA-damaging agents.

Mutant template hTR induces the formation of DNA damage foci and exacerbates the antiproliferative effects of anticancer drugs

We also analysed the cell cycle profile in the vector and the mutant hTR clones derived from the YCC-B2 cells after drug treatment. Vector cells showed a marked decrease in the percentage of cells in G1 and a slight increase in G2/M after 24 h of doxorubicin treatment as indicated by a reduction of the G1/G2–M ratio compared to untreated cells (Figure 5a and data not shown). Also, we detected a slight increase in the sub-G1 fraction after drug treatment (data not shown). These characteristics are indicative of mitotic catastrophe, a process of cell death that occurs during or after mitosis in response to several anticancer agents, such as anthracyclines and taxanes (Castedo *et al*., 2004; Brown and Attardi, 2005). Mitotic catastrophe is characterized by aberrant mitoses and polyploidy, which may be followed by apoptosis (Castedo *et al*., 2004; Brown and Attardi, 2005). Interestingly, in the clonal populations expressing the mutant hTR, the effects of doxorubicin treatment were more pronounced than in the vector cells. The percentage of cells in G1 was further decreased compared to the vector cells, whereas the percentage of cells in G2/M was similar, resulting in a greater reduction of the G1/G2–M ratio (Figure 5a). After 72 h, the main effect of doxorubicin treatment was a marked increase in the percentage of cells in sub-G1 in the vector clone compared to untreated cells, with an even larger increase in the mutant clones (Figure 5b). Thus, the reduction in the number of colonies following drug treatment in the mutant populations could be attributed to an exacerbation of the antiproliferative effects of doxorubicin in the presence of the mutant hTR, resulting in an alteration of the cell cycle profile associated with an initial G2/M accumulation and high levels of cell death.

A previous study has shown that expression of high levels of mutant template hTRs induces a DNA damage response with the formation of nuclear foci at the telomeres that colocalize with the DNA damage protein 53BP1 (Xu and Blackburn, 2004). In order to understand whether that was the case also in cells expressing low levels of the mutant hTR, we monitored the presence of 53BP1 foci in vector and MuA-hTR YCC-B2 clones. We found that the mutant populations had a significantly higher percentage of cells containing 53BP1 foci compared to the vector cells, although only few foci/cell were detected (1–4 foci/cell; Figure 5c and data not shown). As expected, following doxorubicin treatment both the percentage of foci-containing cells and the number of foci/cell were increased in all populations analysed, whereas treatment with paclitaxel did not affect either parameter (data not shown). These results suggest that the presence of the mutant template hTR causes a slight disturbance of the telomere structure, which results in the formation of DNA damage foci; given the low level of MuA-hTR expression and the presence of the wild-type enzyme, these effects are mild and do not affect cell viability. When cells are treated with anticancer drugs, however, these disturbances of the telomere cap exacerbate the antiproliferative effects of the drugs.

Mutant hTR increases drug sensitivity in immortal cells that maintain telomeres via the ALT pathway

A total of 10–15% of human tumors do not rely on telomerase for telomere maintenance but use an

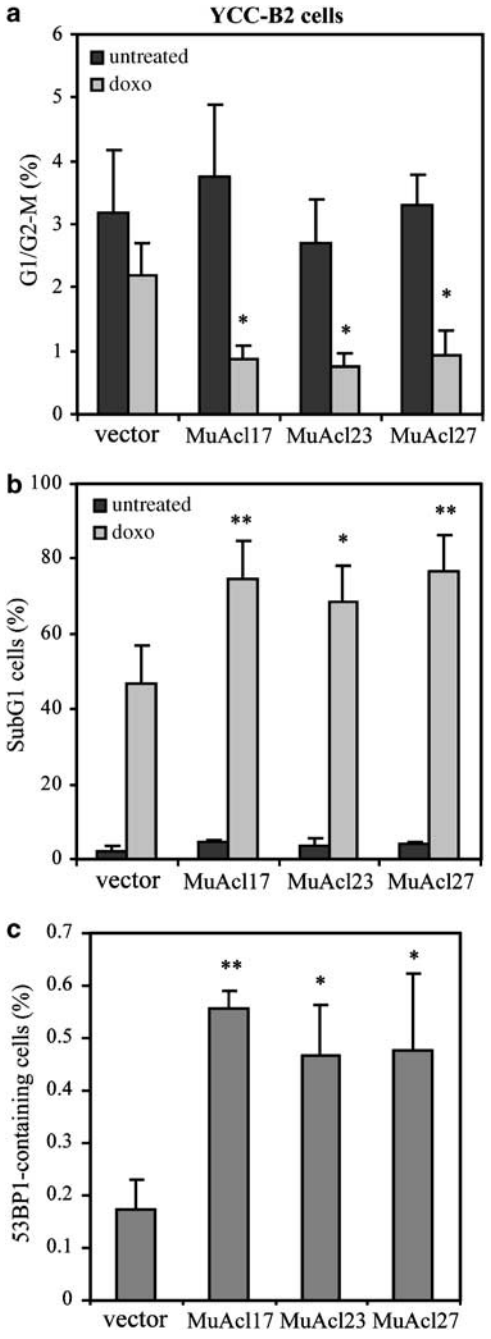

Figure 5 Doxorubicin treatment alters the cell cycle profile in MuA-hTR-expressing YCC-B2 cells. (**a**) The cell cycle profile of one vector and three mutant hTR clones was analysed 1 day after doxorubicin treatment and the values of the ratio between the percentage of cells in G1 and in G2–M are indicated. As a comparison, the G1/G2–M ratios in the untreated cells are shown. (**b**) The percentage of sub-G1 cells was analysed in vector and mutant hTR clones either left untreated or treated for 3 days with doxorubicin. (**c**) MuA-hTR induces the formation of 53BP1 foci. The percentage of cells containing 53BP1 foci (1–4 foci/cell) in the vector and mutant hTR YCC-B2 clones is shown. Values represent the means $\pm$ s.d. of at least three independent experiments. Statistical differences between the vector and each mutant clone were calculated with the unpaired *t*-test and are indicated as $*P<0.05$ and $**P<0.01$.

alternative mechanism most likely based on recombination (Bryan *et al*., 1997; Dunham *et al*., 2000). We sought to investigate whether drug treatment combined with telomere destabilization caused by the mutant hTR could be exploited as a general approach for various tumor cells regardless of the mechanism used to maintain their telomeres. For this purpose, we chose an ALT cell line, GM847, which does not use telomerase to maintain telomere length and expresses only hTR. Telomerase can be reconstituted in these cells by reintroducing the catalytic subunit hTERT (Wen *et al*., 1998). GM847 cells were transfected with a plasmid encoding hTERT alone or hTERT and MuA-hTR together to reconstitute a wild-type or a mutant telomerase, respectively (Figure 6). We reasoned that if the synthesis of mutant telomeric repeats disturbed the telomere cap, the mutant cells should be more sensitive to drug treatment than the controls although they can maintain telomere length through the ALT pathway. Indeed colony-forming assays of doxorubicin-treated GM847 cells expressing hTERT and MuA-hTR yielded fewer colonies compared to both the vector clone and clones expressing wild-type telomerase (Figure 6d), indicating that the presence of hTERT and the mutant RNA sensitizes ALT GM847 cells to doxorubicin treatment. Moreover, these effects did not require changes in overall telomere length (Figure 6c).

Discussion

Telomere maintenance is an essential requisite for cell proliferation. Disturbing telomere integrity results in impairment of cell proliferation and loss of viability. Several reports have validated telomerase as a possible therapeutic target for cancer treatment (reviewed by Kelland, 2005). However, targeting telomerase alone or in combination with anticancer drugs is not sufficient to trigger rapid death of all tumor cells owing to the lag phase necessary for telomeres to become critically short and dysfunctional (Kelland, 2005). In contrast, disturbances of the telomeric capping induce cell growth arrest without significant telomere shortening and therefore may act more rapidly (Karlseder *et al*., 1999; Takai *et al*., 2003). In this study, we analysed the effects of drug treatment in cells in which we interfered with telomere structure by the introduction of a mutant template hTR. We show that disturbing telomere structure significantly increased the sensitivity of human tumor cells to a variety of anticancer drugs.

We report that cells in which telomere disturbances were induced through the introduction of a mutant template hTR did not undergo overall telomere shortening, although broader telomere length distributions within cells were apparent. The presence of the mutant hTR did not significantly affect the ability of the cells to proliferate both in mass culture and when plated at limiting dilutions nor did induce any change in the cell cycle profile. Nonetheless, cells expressing the mutant RNA were more sensitive to treatment with anticancer

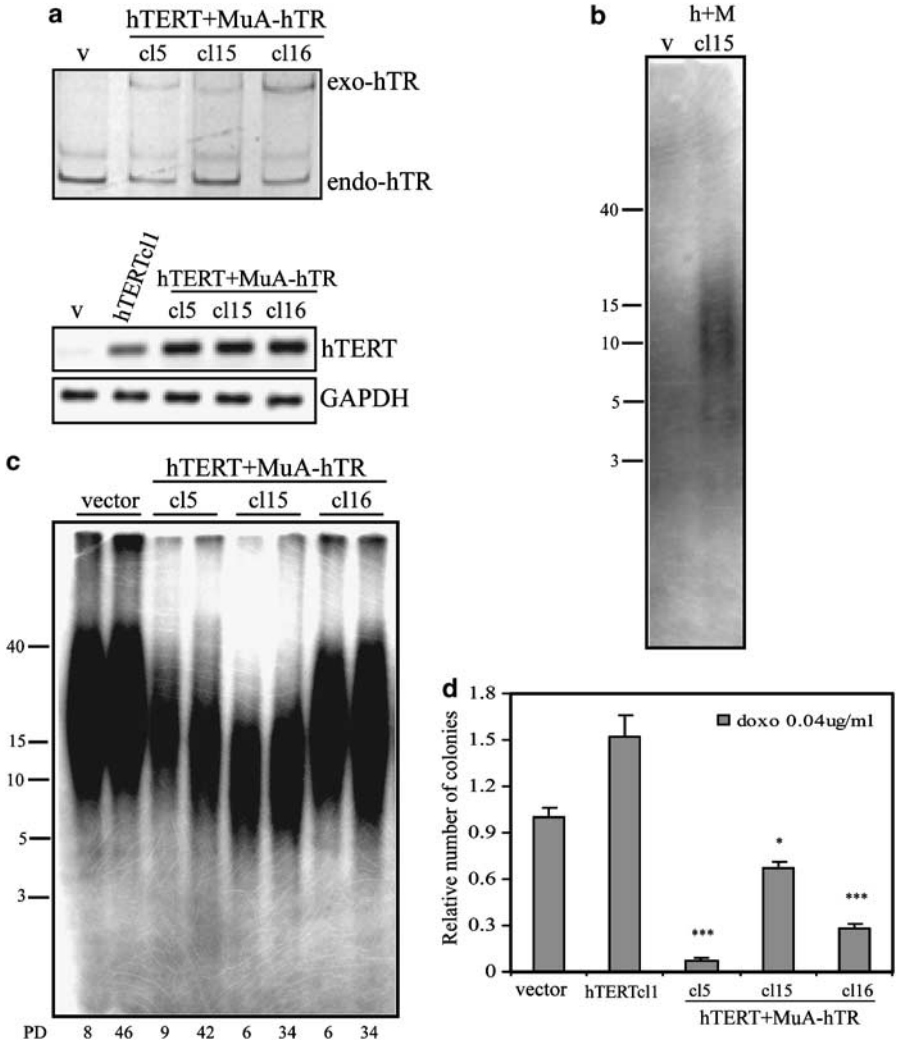

Figure 6 Reconstitution of a mutant telomerase enzyme sensitizes GM847 cells to anticancer drugs. (**a**) One vector-, one hTERT- and three hTERT + hTR-MuA-expressing clones were analysed by RT–PCR for the expression of MuA-hTR (upper panel; exo-hTR, exogenous mutant hTR; endo-hTR, endogenous wild-type hTR) and hTERT (lower panel). GAPDH was used as a control. (**b**) The introduction of the mutant hTR results in incorporation of mutated repeats onto the telomeres. TRF analysis of one vector- and one hTERT + hTR-MuA-expressing clone (h + M cl15) was monitored with a MuA-specific telomeric probe. Representative molecular weight markers are indicated. (**c**) Expression of MuA-hTR does not have any effect on telomere length maintenance. TRF lengths of the clones shown in (**a**) were analysed by PFGE at early and late PDs. Representative molecular weight markers are indicated. (**d**) Colony-forming assay of the same clones as in (**a**) treated with the indicated concentrations of doxorubicin (doxo). The relative numbers of colonies were obtained as a ratio of the colonies in the mutant clones to the colonies in the vector clone. Values ± s.e.m. of at least three independent experiments are shown. Statistical differences between the vector and each mutant clone were calculated with the unpaired *t*-test. $*P<0.05$; $***P<0.001$.

drugs compared to the controls. The response to drug treatment required an active telomerase enzyme and did not depend on the p53 status of the cells. Cell cycle analyses of YCC-B2 vector and mutant template hTR clones showed an alteration of the cell cycle profile after drug treatment, with a marked decrease in the percentage of the cells in G1 and an increase in the sub-G1 fraction, which was more pronounced in the mutant clones than in the vector cells. These changes are indicative of cell death by mitotic catastrophe. Previous studies in human ALT cells and in lower eucaryotes have shown that mutated telomeres have deleterious effects on cell viability and result in cell cycle abnormalities and cell death by mitotic catastrophe (Yu *et al*., 1990; Kirk *et al*., 1997; Smith and Blackburn, 1999; Guiducci *et al*., 2001; Lin *et al*., 2004). Thus, although the expression of MuA-hTR does not affect cell viability in normal growth conditions, it may enhance the antiproliferative effects of doxorubicin, resulting in higher drug sensitivity compared to control cells.

Although the aim of this work was not to characterize the mechanism by which mutated telomeric repeats increase cell sensitivity to anticancer drugs, we speculate that the presence of mutant sequences affects the binding of the shelterin complex, disturbs telomere

capping and induces a DNA damage response. A recent study has shown that high levels of mutant template hTRs induce the formation of nuclear foci at the telomeres that colocalize with DNA damage proteins, such as 53BP1 (Xu and Blackburn, 2004). Similar to these findings, we found a higher percentage of cells containing 53BP1 foci in the mutant clones compared to the vector cells. However, we did not observe any effect of the mutant hTR on cell viability and proliferation, possibly owing to the lower levels of MuA-hTR expression. Nevertheless, the presence of mutated repeats is likely to disturb the telomere capping thereby exacerbating the antiproliferative effects of anticancer drugs. It has recently been proposed that telomerase has other yet uncharacterized telomere-independent functions besides telomere length maintenance (Chung *et al*., 2005). Although we cannot exclude the possibility that the mutant hTR may interfere with these alternative functions of telomerase, it seems very likely that the ability to synthesize new telomeric repeats is essential for the mutant hTR-dependent effects.

Our results obtained using the combination of mutant template hTR and chemotherapeutic drugs differ significantly from those reported with telomerase inhibition-based approaches described to date. The main difference is that the increased sensitivity to anticancer drugs imparted by the mutant hTR does not require overall telomere shortening, eliminating the lag phase associated with telomerase inhibition. The observation that all the clonal derivatives expressing the mutant RNA were more sensitive to drug treatment than the controls independently of their initial telomere lengths supports this conclusion. Indeed, cells with long telomeres (YCC-B2) and cells with much shorter telomeres (YCC-B1) responded similarly to the drugs. Moreover, we found that reconstitution of a mutant telomerase enzyme in immortal ALT cells with extremely long telomeres resulted in greater sensitivity to chemotherapeutic drugs compared to control cells, without any obvious effect on telomere length maintenance or cell proliferation. These results confirm that disturbing the telomeres in a telomerase-dependent manner can be used to sensitize immortal ALT cells to anticancer drugs. Another important difference between our study and previous telomerase-based anticancer approaches is that cells expressing the mutant hTR became more sensitive to anticancer drugs with different mechanisms of action. Several reports have indicated that telomerase inhibition sensitizes cells specifically to agents that induce DNA breaks, but has no effect with drugs that act through other mechanisms (Lee *et al*., 2001; Sharma *et al*., 2003; Shin *et al*., 2004; Masutomi *et al*., 2005; Nakamura *et al*., 2005). However, we found that YCC-B2 cells expressing MuA-hTR are also more susceptible than control cells to treatment with paclitaxel, a widely used chemotherapeutic that acts primarily by stabilizing the microtubules of the mitotic spindle, excluding specificity for DNA-damaging agents at least in the context of the mutant hTR.

Altogether, our data indicate that interfering with telomere structure in cancer cells through the introduction of mutant template hTR could be an effective and general strategy to block tumor cell growth. More importantly this approach may lead to the development of a clinical therapy for the treatment of all tumors independently of their initial telomere lengths and mechanisms to maintain them. This could allow the use of lower levels of chemotherapeutics or shorter treatment time thereby reducing systemic cytotoxicity.

Materials and methods

Cell lines and plasmids

YCC-B1 and YCC-B2 breast cancer cells were kindly provided by Dr Sun Young Rha of the Cancer Metastasis Center, Yonsei University College of Medicine, Korea (Park *et al*., 1998) and were grown in minimum essential medium (MEM) with 10% heat-inactivated fetal bovine serum (FBS, Wisent). MCF-7 cells were obtained from Dr Pollack (Lady Davis Institute, Montreal, Canada) and were grown in RPMI with 10% FBS. GM847 and VA13 cells obtained from Dr Silvia Bacchetti (Regina Elena Cancer Institute, Rome, Italy) were grown in α-MEM with 10% FBS.

phTR and phTR-MuA containing, respectively, wild-type hTR and a mutant hTR specifying TTTGGG telomeric sequences (Marusic *et al*., 1997; Guiducci *et al*., 2001) driven by the endogenous hTR promoter, and phTERT containing wild-type hTERT and the puromycin resistance gene were obtained from Dr Silvia Bacchetti. phTERT/hTR-MuA was obtained by subcloning hTR-MuA into the phTERT plasmid.

Transfections

Transfections of breast cancer cells were performed by DNA-calcium phosphate with the vector or phTR-MuA. Stable clonal populations were selected with 0.25 μg/ml puromycin for 10–14 days. GM847 cells were transfected with either phTERT or phTERT/hTR-MuA and selected in 0.2 μg/ml puromycin. VA13 cells were transfected with either the vector or phTR-MuA and selected in 0.3 μg/ml puromycin. All cell populations were routinely subcultured at a 1:4 split ratio as they reached confluence. Population doublings (PDs) were calculated taking as PD 0 the time when clones first reached confluence in a 60 mm plate.

Telomerase assay and Western blot analysis

Whole-cells extracts were prepared by detergent lysis and assayed by the PCR-based TRAP (Kim *et al*., 1994) using serial dilutions of the extracts. For the MuA-hTR-expressing clones, TRAP assay was performed using 5–7 μg of protein extracts with PCR conditions and reverse primers specific for the mutant hTR as described previously (Feng *et al*., 1995). Western blot analyses were performed using 40 μg of protein extracts. The following antibodies were used: anti-mouse p53 (Ab6, Oncogene Science, kindly provided by Dr Koromilas, Lady Davis Institute, Montreal, Canada), anti-mouse p21 (Upstate, Lake Placid, NY, USA) and anti-mouse β-actin (MA1501, Chemicon, Temecula, CA, USA). Secondary antibodies were purchased from Sigma, Oakville, ON, USA and used according to the manufacturer's instructions.

DNA analysis

For TRF analysis, DNA was extracted using standard procedures, digested with *Hin*fI/*Rsa*I and separated by pulse

field gel electrophoresis (PFGE; Bryan *et al*., 1995). The gel was then denatured, neutralized and partially dried. Wild-type telomeric sequences were detected by hybridization with a [γ-^{32}P]dATP 5′-end-labeled telomeric probe $(C3TA2)_3$, whereas mutant telomeric sequences were detected using a [γ-^{32}P]dATP 5′-end-labeled mutant-specific probe $(C3A3)_4$. pBSdT34, containing nine TTTGGG repeats, was used as a positive control for hybridization with the mutant probe. After hybridization, gels were exposed to Phosphoimager. Hybridization signals were quantified with ImageQuant (Molecular Dynamics, Sunnyvale, CA, USA) as described (Harley *et al*., 1990; Bryan *et al*., 1995).

G-tail length was analysed with the T-OLA assay (telomere-oligonucleotide ligation assay) as described previously (Cimino-Reale *et al*., 2001; Stewart *et al*., 2003). Genomic DNA (5 μg) was hybridized with 0.5 pmol of [γ-^{32}P]dATP 5′-end-labeled telomeric probe at 50°C overnight, ligated for 5 h, precipitated and separated on 5% acrylamide, 6 M urea gel. As a loading control, 10 ng of the ligated DNA was used for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplification.

RT–PCR

RNA was isolated using TRIzol (Invitrogen, Burlington, ON, USA) according to the manufacturer's instructions. The following primers were used:

For wt-TR and MuA-hTR: LX, 5′-GAGAGAGTGACTCT CACGAGAGCC-3′; F3B, 5′-TCTAACCCTAACTGAGAA GGGCGTAG-3′; and R3C, 5′-GTTTGCTCTAGAATGAAC GGTGGA-3′. The LX and R3C primers recognize the transfected hTR, whereas F3B and R3C primers recognize the endogenous hTR.

For hTERT: hT1, 5′-AAGTTCCTGCAGTGGCTGAT GAG-3′ and hT5, 5′-TCGTAGTTGAGCACGCTGAACAG-3′.

For human GAPDH: RT11, 5′-CGGAGTCAACGGATTT GGTCGTAT-3′ and RT12, 5′TGCTAAGCAGTTGGTGGT GCAGGA-3′.

Q-FISH and immunofluorescence

Metaphase chromosome spreads were prepared from vector- or MuA-hTR-expressing YCC-B2 cells as described previously (Cerone *et al*., 2001). Fixed cells were hybridized with a telomeric (C3TA2)-Cy3 PNA probe and counterstained with 4,6,diamidino-2-phenylindole and fluorescent signals were captured using a CCD camera (Photometrics-Sensys, Tucson, AZ, USA). Original black and white Cy3 images were used for quantitative analysis using the Iplab Spectrum P Software. To obtain telomere relative intensities, the mean pixel value of each telomere was divided by the mean telomere intensity of the metaphase.

For immunofluorescence, vector and MuA-hTR YCC-B2 cells were fixed with 2% paraformaldehyde for 15 min, permeabilized with 0.25% Triton X-100 for 5 min and blocked in phosphate-buffered saline (PBS) with 5% FBS for 1 h at room temperature. 53BP1 foci were detected with a mouse monoclonal antibody against 53BP1 (Upstate Cell Signaling Solutions, clone BP13) at 1:100 dilution, followed by fluorescein isothiocynate-conjugated goat anti-mouse secondary antibody (Jackson Immuno Research, West Grove, PA, USA) at 1:200 dilution. Nuclei were stained with 0.5 μg/ml Hoechst 22358 and cells were analysed using an Olympus BX51 fluorescence microscope. Between 35 and 150 cells were counted in each experiment. Statistical differences were analysed by the unpaired *t*-test using the online GraphPad QuickCalcs software and statistical significance is expressed as $*P<0.05$ and $**P<0.01$. The experiments were repeated at least three times.

Drug treatments and cell viability

Doxorubicin, etoposide and paclitaxel were purchased from Sigma. For the 3-[4,5] dimethylthiazole-2,5-diphenyltetrazolium bromide (MTT) assay, cells were seeded at $5\times10^3-10^4$/well in 0.2 ml in 96-well plates. After 24 h, cells were treated with increasing concentrations of the drugs for 48 h. At the end of the experiment, the MTT assay was performed as described (Christodoulopoulos *et al*., 1999). The IC_{50} was calculated as the concentration of the drugs that resulted in 50% reduction of cell viability compared to untreated controls.

For colony-forming assays in the absence of drug, 2.5–5×10^3 cells/6-well plates were seeded, incubated for 48 h and plated at low density in 10 cm plates in triplicate to allow colony formation. After 14–21 days, the colonies were stained with crystal violet and counted. For drug treatment, YCC-B1, YCC-B2, GM847 and VA13 derivatives were seeded at 10^5 cells/6-well plates, whereas MCF-7 derivatives were seeded at 2.5×10^5 cells/6-well plates. The next day, cells were treated with the indicated concentrations of drugs for 24 h and then plated at low density in 10 cm plates in triplicate until colonies were visible and could be stained with crystal violet and counted. For accuracy, experiments resulting in less than 25 and more than 1500 colonies in the controls were not used. Relative numbers of colonies were calculated as a ratio between the numbers of colonies in the mutant hTR clones and the number of colonies in the vector clones. Comparisons between vector cells and each derivative were analysed by the unpaired *t*-test using the online GraphPad QuickCalcs software, and statistical significance is expressed as $*P<0.05$, $**p<0.01$ and $***P<0.001$. All experiments were repeated at least three times.

Cell cycle analysis

For cell cycle analysis, cells were either left untreated or treated with doxorubicin as described above. Samples were collected after 1 and 3 days of treatment and fixed with ice-cold ethanol. Before analysis, cells were centrifugated, resuspended in 500 μl of cold PBS containing 200 μg/ml of RNAse A and incubated overnight at 4°C. The following day, cells were incubated for 15 min with 25 μg of propidium iodide and the DNA content was measured by flow cytometry (Becton Dickinson, San Jose, CA, USA, fluorescence activated cell sorter, FACS), followed by quantification with CellQuest software. For each sample, at least 20 000 events were collected. The experiments were repeated at least three times. Statistical differences between vector cells and each mutant hTR clone were analysed by the unpaired *t*-test using the online GraphPad QuickCalcs software and are expressed as $*P<0.05$ and $**P<0.01$.

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Telomerase inhibition enhances the response to anticancer drug treatment in human breast cancer cells

Maria Antonietta Cerone,[1,2] J. Arturo Londoño-Vallejo,[3] and Chantal Autexier[1,2]

[1]Department of Anatomy and Cell Biology and [2]Bloomfield Centre for Research in Aging, Lady Davis Institute, Jewish General Hospital, Montreal, Quebec, Canada; and [3]Telomeres and Cancer Laboratory, Pavillion Trouillet-Rossignol, Institut Curie, Paris, France

Abstract

Breast cancer is the most common malignancy among women. Current therapies for breast tumors are based on the use of chemotherapeutic drugs that are quite toxic for the patients and often result in resistance. Telomerase is up-regulated in 95% of breast carcinomas but not in adjacent normal tissues. Therefore, it represents a very promising target for anticancer therapies. Unfortunately, the antiproliferative effects of telomerase inhibition require extensive telomere shortening before they are fully present. Combining telomerase inhibition with common chemotherapeutic drugs can be used to reduce this lag phase and induce tumor cell death more effectively. Few studies have analyzed the effects of telomerase inhibition in combination with anticancer drugs in breast cancer cells. In this study, we inhibited telomerase activity in two breast cancer cell lines using a dominant-negative human telomerase reverse transcriptase and analyzed cell viability after treatment with different anticancer compounds. We found that dominant-negative human telomerase reverse transcriptase efficiently inhibits telomerase activity and causes telomere shortening over time. Moreover, cells in which telomerase was suppressed were more sensitive to anticancer agents independently of their mechanism of action and this sensitization was dependent on the presence of shorter telomeres. Altogether, our data show that blocking telomere length maintenance in combination with anticancer drugs can be used as an effective way to induce death of breast cancer cells. [Mol Cancer Ther 2006;5(7):1–7]

Introduction

A hallmark of cancer cells is unlimited cell proliferation, which requires the ability to maintain telomere length during cell division. Telomeres are essential structures that cap the ends of eukaryotic chromosomes and are made of short G-rich DNA repeats associated with specific proteins (1). Their main function is to protect the chromosome ends from being recognized as DNA breaks by the DNA repair machinery (2). Another important function of the telomeres is to buffer the loss of terminal sequences due to the end replication problem, which results in DNA shortening at each round of cell replication (3, 4). When telomeres become critically short, cells stop dividing and enter replicative senescence (5). This irreversible growth arrest acts as a powerful tumor suppressor mechanism to block proliferation of cells in which dysfunctional telomeres may induce genome instability and therefore malignant transformation (6). Cells with unlimited proliferative potential, such as germ line, stem cells, and cancer cells, activate mechanisms to ensure the maintenance of telomere length. Most human cells engage telomerase, an RNA-dependent DNA polymerase with a reverse transcriptase subunit [called human telomerase reverse transcriptase (hTERT)], which uses its intrinsic RNA subunit (called hTR) as a template for synthesis of new telomeric repeats at the chromosome termini. Telomerase is mostly inactive in normal adult human cells, with the exception of germ line and stem cells, whereas it is reactivated in over 90% of human cancers (7–9). The almost universal presence of telomerase in human tumors suggests that targeting telomerase may represent an efficient way to specifically block tumor cell growth with minor effects on normal cells. Several approaches have been developed to block the activity of the telomerase holoenzyme, such as antisense oligonucleotides against either hTERT or hTR (10–12), inactive variants of hTERT that act as dominant negatives (13, 14), small chemical compounds against hTERT (15–18), and G-quadruplex-stabilizing agents that bind the telomeric ends and block telomerase access and elongation (19–21). In all cases, direct or indirect telomerase inhibition resulted in the inability of the cells to maintain telomere length and ultimately cell growth arrest or cell death. However, these effects were not immediately observed due

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Requests for reprints: Chantal Autexier, Bloomfield Centre for Research in Aging, Lady Davis Institute, Sir Mortimer B. Davis Jewish General Hospital, 3755 Cote Ste Catherine, Montreal, Quebec, Canada H3T 1E2. Phone: 514-340-8260; Fax: 514-340-8295. E-mail: chantal.autexier@mcgill.ca

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to the requirement for extensive telomere shortening. Combinations of telomerase inhibition and anticancer drugs have been used to reduce this lag phase and induce cell death more rapidly. Indeed, several reports have shown that these approaches may be more effective in killing tumor cells than telomerase inhibition alone, although in most cases they still depend on telomere shortening (11, 15, 22–27). Moreover, the effects of combination approaches were often cell line and drug type specific (11, 23, 25, 26).

Breast cancer is one of the most common malignancies among women. Typically after surgical removal of the tumor mass, breast cancer patients are treated with chemotherapeutic drugs that are quite toxic, lack selectivity, and often result in resistance. Estrogen-responsive tumors that initially respond to tamoxifen often develop resistance to this agent and progress to metastatic disease. The new generations of aromatase inhibitors, which have been shown to be highly effective in early breast cancer settings, also eventually induce resistance (28). Therefore, more effective treatments are needed to treat breast cancer. Telomerase is up-regulated in 95% of breast carcinoma, but not in adjacent normal tissues, and its activity increases with tumor aggressiveness (29–33). Few studies have analyzed the effects of telomerase inhibition in combination with anticancer drugs in breast cancer cells (15, 25, 27). In this study, we inhibited telomerase activity in two telomerase-positive breast cancer cell lines through the introduction of a dominant-negative hTERT (dn-hTERT) variant and analyzed the effects on cell viability. We also measured cell survival and proliferative ability in the presence of anticancer agents commonly used for chemotherapy. We found that telomerase inhibition induces telomere shortening over time and affects cell viability in a telomere length–dependent manner. Moreover, cells in which telomerase was suppressed were more sensitive than the controls to a variety of anticancer drugs. Altogether, our data confirm that a combination approach based on telomerase inhibition and anticancer drugs could be used to effectively induce death of human breast cancer cells.

Materials and Methods

Cell Culture and Plasmids

YCC-B2 breast cancer cells (provided by Dr. Sun Young Rha, Cancer Metastasis Center, Yonsei, Korea; ref. 34) were grown in MEM with 10% heat inactivated fetal bovine serum (Wisent). MCF-7 cells (obtained from Dr. Pollack, Lady Davis Institute, Montreal, Canada) were grown in RPMI with 10% fetal bovine serum.

phTERT containing wild-type hTERT and the puromycin resistance gene was obtained from Dr. Silvia Bacchetti (Regina Elena Cancer Institute, Rome, Italy). pdnhTERT, encoding for a dn-hTERT (D868N), was generated by site-specific mutagenesis (QuickChange Site-Directed Mutagenesis kit, Stratagene) using phTERT as template and the following primers: 5′-CTCCTGCGTTTGGTTAACGATTTCTTGTTG-′3 and 5′-CAACAAGAAATCGTTAACCAAACGCAGGAG-′3 (35). The mutation was confirmed by sequence analysis and by digestion with *Hin*cII, a restriction site created by the mutation.

YCC-B2 and MCF-7 cells were transfected with the vector or dn-hTERT by DNA-calcium phosphate and stable clonal populations were selected with 0.25 μg/mL puromycin for 1 to 2 weeks. All cell populations were routinely subcultured at a 1:4 split ratio as they reached confluence. Population doubling (PD) 0 was defined as the time when clones first reached confluence in a 60 mm plate.

Telomerase Activity (Telomeric Repeat Amplification Protocol) Assay and Western Blot

Whole cells extracts were prepared by detergent lysis and assayed by the PCR-based telomeric repeat amplification protocol (36) using 50 and 100 ng extracts. Telomerase activity in the dn-hTERT derivatives was quantified relative to the internal PCR control and expressed as a percentage of the activity measured in the vector cells. Western blot analyses were done using 50 μg protein extracts. The following antibodies were used: antimouse estrogen receptor-α (MAB461, Chemicon International, kindly provided by Dr. Miller, Lady Davis Institute, Montreal, Canada) and antimouse β-actin (MA1501, Chemicon International). Secondary antibodies were purchased from Sigma.

Telomere Length Analysis

For telomere restriction fragment analysis, DNA was extracted using standard procedures, digested with *Hin*fI/*Rsa*I and separated by pulse field gel electrophoresis (37). The gel was denatured, neutralized, partially dried, and hybridized with a [γ-^{32}P]dATP 5′ end-labeled telomeric probe $(C3TA2)_3$ (38). After hybridization, gels were exposed to Phosphoimager, hybridization signals were quantified with ImageQuant (Molecular Dynamics), and telomere length was calculated according to the formula $\Sigma(OD_i)/\Sigma(OD_i/L_i)$, where OD_i indicates the absorbance at the position i, and L_i is the molecular weight marker at the same position (37, 38).

Quantitative Fluorescence *In situ* Hybridization Analysis

Quantitative fluorescence *in situ* hybridization was done as previously described (38). Briefly, metaphase chromosome spreads were prepared from vector- and dn-hTERT–expressing YCC-B2 cells, fixed, hybridized with a telomeric (C3TA2)-Cy3 PNA probe, and counterstained with 4′,6-diamidino-2-phenylindole. Fluorescent signals were captured using a charge coupled device camera (Photometrics-Sensys) and quantified using the Iplab Spectrum P Software. To obtain telomere relative intensities, the mean pixel value of each telomere was divided by the mean telomere intensity of the metaphase.

Reverse Transcription-PCR

RNA was isolated using TRIzol (Invitrogen) and the expression of dn-hTERT was analyzed by PCR with the following primers: dn2600, 5′-GGGTTTGGTTAAGGATTTC-3′, and hTERT3141, 5′-TCAGGATGGAGTAGCAGAG-3′.

As a control for RNA integrity, human glyceraldehyde-3-phosphate dehydrogenase was amplified using RT11,

5′-CGGAGTCAACGGATTTGGTCGTAT-3′, and RT12, 5′TGCTAAGCAGTTGGTGGTGCAGGA-3′.

Colony-Forming Assays and Drug Treatment

For colony-forming assays of untreated cells, MCF-7 and YCC-B2 derivatives were seeded at 2.5×10^3 to 5×10^3 cells/six-well plates, incubated for 48 hours, and plated at low density in 10 cm plates in triplicates to allow colony formation. After 2 weeks, colonies were stained with crystal violet and counted. Drug concentrations used for the colony-forming assays were determined through the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, done as previously described (15, 39). For colony-forming assay, after drug treatment, YCC-B2 and MCF-7 derivatives were seeded at 10^5 and 2.5×10^5 cells/six-well plates, respectively. The next day, cells were treated with the indicated concentrations of doxorubicin, etoposide, or paclitaxel (all purchased from Sigma) for 24 hours and plated at low density in 10 cm plates in triplicate until colonies were clearly visible (diameter >50 μm) and ready to be stained with crystal violet. For accuracy, experiments resulting in <25 colonies in the controls were not counted. Relative numbers of colonies were calculated as a ratio between the numbers of colonies in the dn-hTERT clones and the number of colonies in the vector clones. All experiments were repeated at least thrice.

Statistical Analysis

Data were graphed using Microsoft Excel. Comparisons between vector cells and dn-hTERT clones were analyzed by the unpaired *t* test using the online GraphPad QuickCalcs software and statistical significance is expressed as $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. The correlation between proliferative ability and number of PDs in MCF-7 cells expressing dn-hTERT was calculated using a multiple regression test and significance of r^2 values was determined with an *F* test (OriginPro 7.5 software).

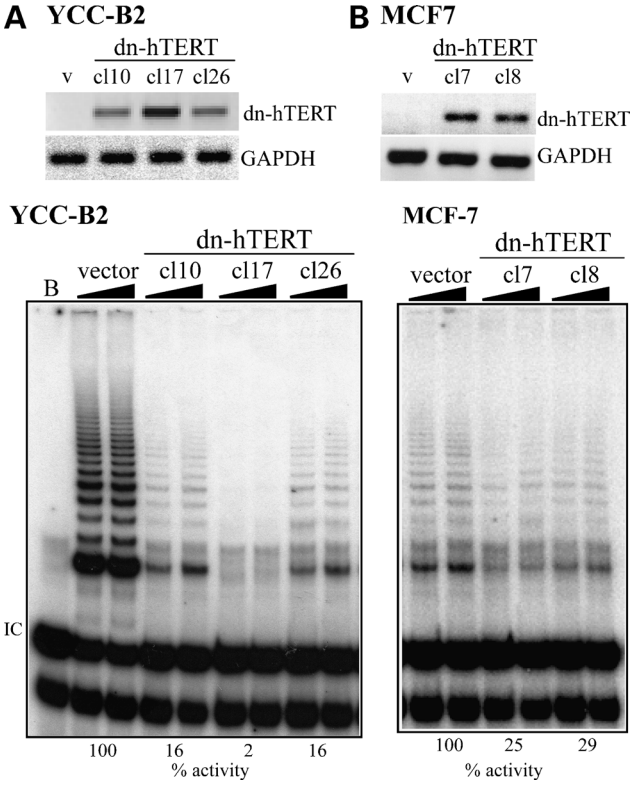

Figure 1. dn-hTERT inhibits telomerase activity in two breast cancer cell lines. The expression of dn-hTERT was analyzed by reverse transcription-PCR in YCC-B2 (**A**, *top*) and MCF-7 (**B**, *top*) derivatives. Telomerase activity of the clonal populations from YCC-B2 (**A**, *bottom*) and MCF-7 (**B**, *bottom*) cells was assayed by telomeric repeat amplification protocol. In all cases, 50 and 100 ng extracts were used. Percentage activity represents the amount of telomerase activity measured in each clone as a percentage of the vector clones (*cl*). *IC,* internal control used for quantification. *B,* blank.

Results

dn-hTERT Inhibits Telomerase Activity and Induces Telomere Shortening

To analyze the effects of telomerase inhibition in breast cancer cells, we used two telomerase-positive cell lines, YCC-B2 and MCF-7. MCF-7 cells maintain stable telomeres of ~7 kb in length, express wild-type p53, and have a functional estrogen receptor-α; YCC-B2 cells have an average telomere length of 10 kb, contain an inactive p53, and do not express the estrogen receptor-α (data not shown; Supplementary Fig. S1).[4] We inhibited telomerase activity in these cells through the introduction of a dn-hTERT variant (35) and selected clonal populations in puromycin for 1 to 2 weeks. The presence of dn-hTERT was detected by reverse transcription-PCR (Fig. 1A and B, *top*). Telomeric repeat amplification protocol assay analyses showed that the introduction of dn-hTERT resulted in significant inhibition of telomerase activity in both YCC-B2 and MCF-7 derivatives (2–29% of the activity in the vector clones; Fig. 1A and B, *bottom*). The biological effects of dn-hTERT expression and telomerase inhibition were evaluated by monitoring telomere length over time. YCC-B2 and MCF-7 vector clones maintained telomere lengths, whereas dn-hTERT–expressing clones showed a marked decrease in telomere length with increasing PDs (Fig. 2). Telomere shortening was more evident in the dn-hTERT clones derived from MCF-7 cells, which after 90 PDs harbored telomeres of 2.6 kb in length (Fig. 2B). Even at the latest passages analyzed (PD 80–94), the presence of dn-hTERT did not have any effect on cell viability and proliferative ability in mass cultures compared with controls in both cell lines (data not shown), suggesting that, although short, telomeres were still relatively functional and able to protect the chromosome ends. However, colony-forming assays of MCF-7 cells expressing dn-hTERT revealed a progressive reduction of proliferative ability compared with control cells, which correlated with cell division and presence of very short telomeres (Figs. 2 and 3). We did not observe a

[4] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

similar effect in dn-hTERT clones obtained from YCC-B2 cells, most likely due to the more limited telomere shortening compared with MCF-7 derivatives (Supplementary Fig. S2).[4]

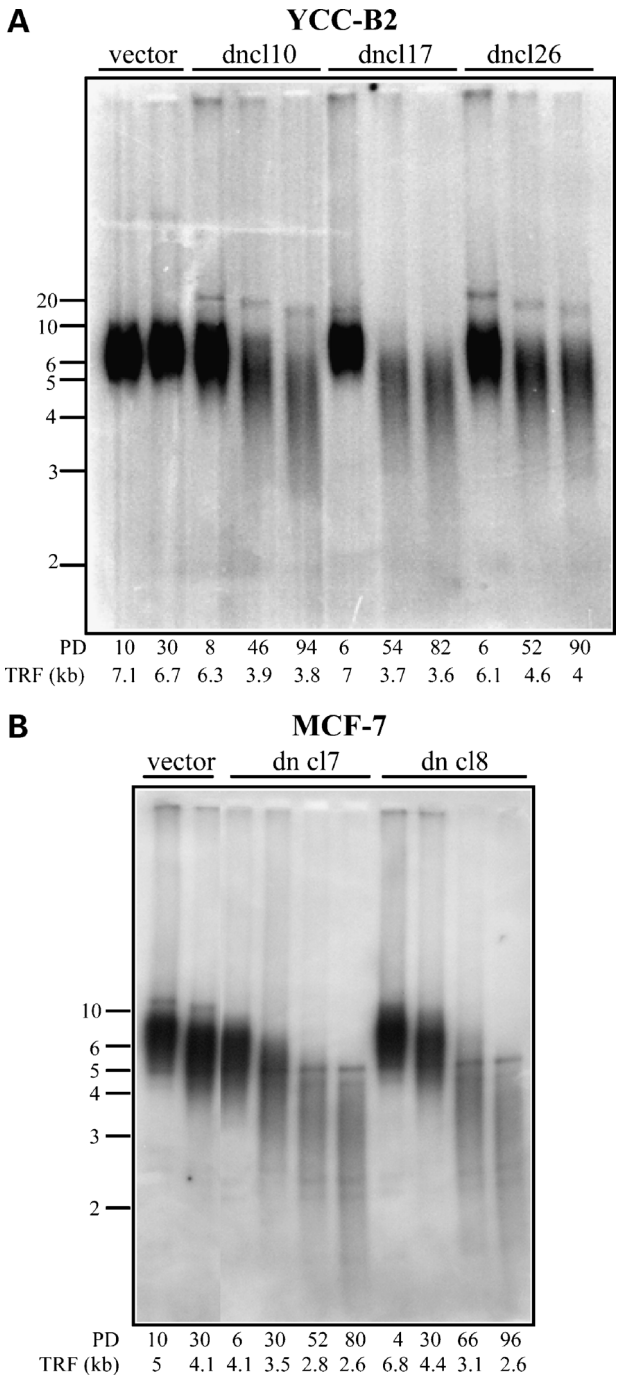

Figure 2. Expression of dn-hTERT induces telomere shortening. Telomere restriction fragment length was analyzed by pulse field gel electrophoresis in YCC-B2 (**A**) and MCF-7 (**B**) vector and dn-hTERT clones at the indicated PDs. Representative molecular weight markers are indicated. The average telomere length was calculated by densitometric scanning of each lane and is shown at the bottom.

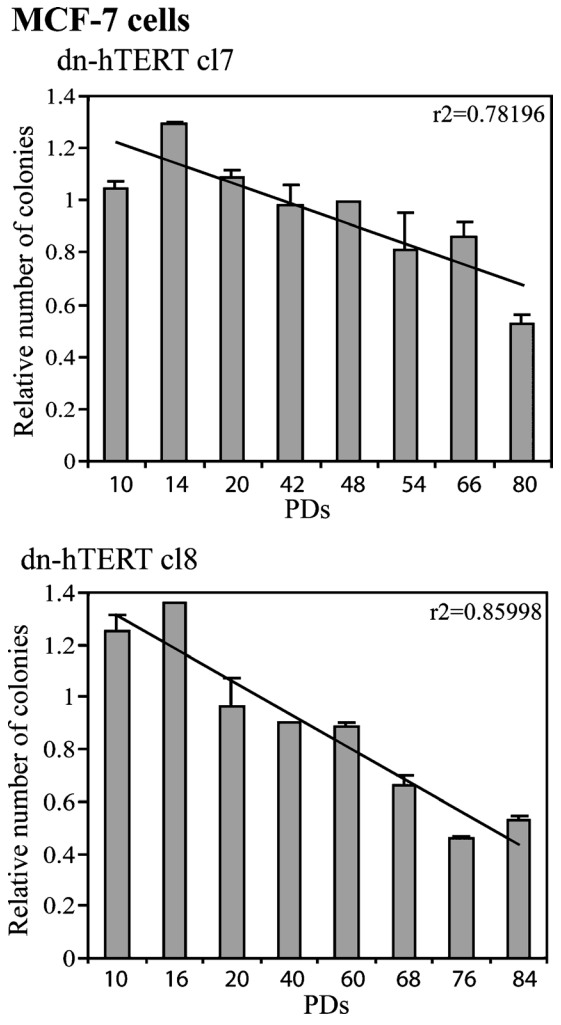

Figure 3. Introduction dn-hTERT in MCF-7 cells induces a progressive reduction of their proliferative ability. Colony-forming assays of MCF-7 dn-hTERT clones 7 and 8 at increasing PDs. The relative numbers of colonies were obtained as a ratio of the colonies in the dn-hTERT clones over the colonies in the vector clones. *Columns,* values of at least three independent experiments; *bars,* SE. r^2 values indicate the correlation between cell proliferative ability and PDs and are both significant with $P < 0.01$.

Increased Sensitivity to Drugs in dn-hTERT–Expressing Clones Requires Telomere Shortening

Previous studies have shown that telomerase inhibition increases the sensitivity of several tumor cell lines to anticancer drugs (11, 15, 22–26). To investigate whether this held true also for breast cancer cells, we treated vector and dn-hTERT–expressing derivatives from MCF-7 and YCC-B2 cells with two DNA-damaging agents used for chemotherapy, etoposide and doxorubicin, and analyzed their ability to survive and proliferate using colony-forming assays. Cells were treated for 24 hours with either doxorubicin or etoposide at concentrations ranging between the IC_{15} and IC_{25} of the parental cells, and subsequently plated at low density and allowed to proliferate for 10 to 14 days until colonies were visible.

Introduction of dn-hTERT in MCF-7 cells induced a significant reduction of the number of colonies compared with the vector cells, indicating that clones expressing dn-hTERT are more sensitive to both doxorubicin and etoposide (Fig. 4A). Similarly, YCC-B2 cells expressing dn-hTERT showed an increased sensitivity to drug treatment compared with the controls (Fig. 4B). Interestingly, these effects were present at early time points after the isolation of the clones (PD 6 and PD 4, in MCF-7 dn-hTERT clones 7 and 8; PD 8 and PD 6 in YCC-B2 dn-hTERT clones 10, 17, and 26). Telomere shortening likely occurred during the selection and the growth of the clones; however, it is technically unfeasible to measure telomere length at the onset or during clone selection. Therefore, we used quantitative fluorescence *in situ* hybridization to analyze the telomere profile in dn-hTERT clones at early and late PDs and compared them with the telomere profile in the controls. We found that the telomere profiles in YCC-B2 dn-hTERT clones 10 and 17 at early passages were broader with a higher frequency of short telomeres when compared with the vector cells, but became narrower at later passages, most likely due to overall telomere shortening (Fig. 5). In addition, although we detected a few chromosome ends without telomeric signals, no obvious increase in telomeric fusions or chromosome instability was seen (Fig. 5; Supplementary Fig. S3),[4] which is in agreement with our data showing that the presence of the dn-hTERT did not significantly affect proliferative ability in YCC-B2 derivatives at the late PDs analyzed (Supplementary Fig. S2).[4] Recent studies have indicated that telomerase elongates preferentially the shortest telomeres, which are more likely to be involved in chromosome aberrations and cause genome instability (40, 41). Therefore, it is possible that in the dn-hTERT–expressing cells, the loss of telomerase affected the shortest telomeres to a greater extent, and this could account for their higher sensitivity to the drugs at early time points compared with control cells.

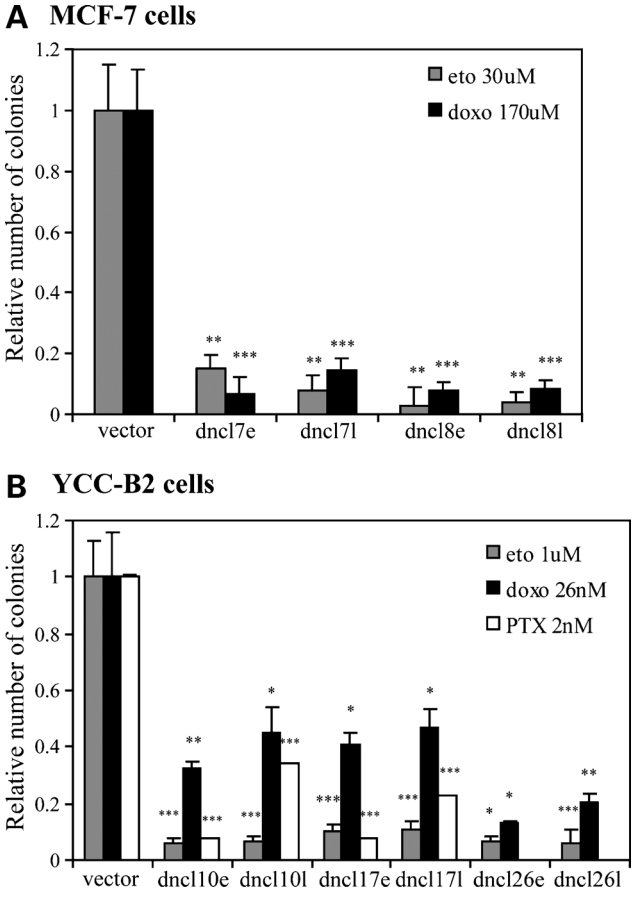

Figure 4. Introduction of dn-hTERT increases the sensitivity of breast cancer cells to chemotherapeutic drugs. **A,** colony-forming assays of vector and dn-hTERT clones from MCF-7 cells treated with the indicated concentrations of doxorubicin (*doxo*) or etoposide (*eto*). **B,** colony-forming assay of YCC-B2 vector and dn-hTERT clones treated with the indicated concentrations of doxorubicin, etoposide, or paclitaxel (*PTX*). The relative numbers of colonies were obtained as a ratio of the colonies in the dn-hTERT clones over the colonies in the vector clones. *Columns,* values of at least three independent experiments; *bars,* SE. Statistical differences between the vector and each dn-hTERT clone were calculated with the unpaired *t* test, using the online GraphPad QuickCalcs software. *e,* early PDs <25; *l,* late PDs >40. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Another chemotherapeutic drug that is largely used for breast cancer chemotherapy belongs to the group of the taxanes, which bind to the microtubules of the mitotic spindle and inhibit segregation of the sister chromatids (42, 43). A recent report showed that telomerase inhibition could sensitize HeLa cells specifically to DNA-damaging agents but not other drugs with different mechanisms of action (11). We therefore analyzed whether suppression of telomerase in YCC-B2 breast cancer cells increased their sensitivity also to this class of anticancer drugs. As shown in Fig. 4B, the number of colonies obtained with two YCC-B2 dn-hTERT–expressing clones (dn-hTERT clones 10 and 17) was significantly reduced compared with the controls after treatment with paclitaxel, excluding a DNA damage–specific response. Our results differ from those obtained in the above study; however, the use of different cell types may account for the discrepancy.

Discussion

Telomerase reactivation is a necessary requirement for the unlimited ability of cancer cells to proliferate. Most human breast tumors analyzed thus far express active telomerase, whereas telomerase cannot be detected in normal adjacent tissues (30, 31). Therefore, it may represent a useful and effective target to induce breast cancer cell death. However, the requirement for telomere shortening before the antiproliferative effects of telomerase inhibition are observed implies that only cells with short telomeres would respond rapidly to such treatments, thus limiting their applicability. More recent reports have indicated that telomerase inhibition could be used to sensitize tumor cells to anticancer drugs (11, 15, 16, 22–26). These combination approaches have the advantage of killing tumor cells more rapidly than either treatment alone and allow the use of lower drug concentrations, thereby reducing cytotoxicity for patients. Although the feasibility of combination approaches has been tested in several tumor cell types,

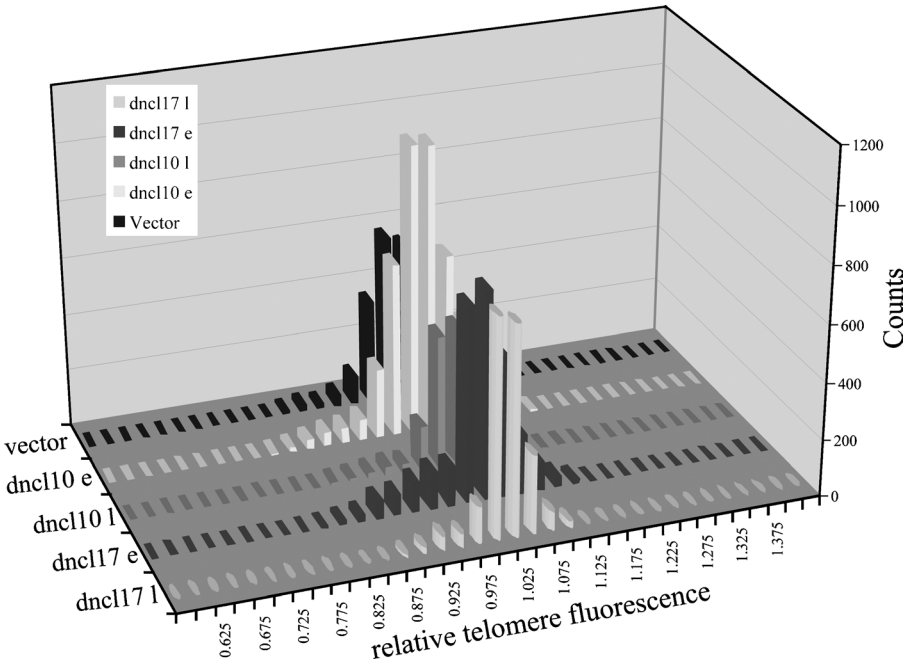

Figure 5. The telomere profile of the vector and two dn-hTERT clones derived from YCC-B2 cells was analyzed by quantitative fluorescence *in situ* hybridization and the distributions of relative telomere fluorescence intensities at early and late PDs are shown. Note the more heterogeneous distributions of relative telomere lengths within cells expressing dn-hTERT at early passages compared with the vector cells.

with different degrees of success dependent on the cell type and the drug analyzed, very few studies have analyzed their effects on breast cancer cells (15, 25).

In this study, we analyzed the effects of telomerase inhibition in combination with anticancer drugs in two breast cancer cell lines with different p53 and estrogen receptor status. We report that breast cancer cells in which telomerase activity was suppressed by dn-hTERT undergo telomere attrition over time with a limited effect on proliferative ability depending on the initial telomere length. More importantly, these cells showed increased sensitivity to different chemotherapeutic drugs compared with control cells, and the sensitization was dependent on the presence of short telomeres. Indeed, telomere restriction fragment and fluorescence *in situ* hybridization analyses revealed higher frequencies of shorter telomeres at early PDs in dn-hTERT clones compared with control cells and marked telomere shortening with successive cell divisions.

Interestingly, telomerase inhibition sensitized breast cancer cells to various drugs with different mechanisms of action. Indeed, YCC-B2 cells in which telomerase was inhibited by dn-hTERT were more sensitive to both DNA-damaging agents, such as etoposide and doxorubicin, and to the microtubule-targeting compound paclitaxel, excluding a DNA damage–specific response. Our results differ from those of a recent study that reported a specific increase in sensitivity to DNA-damaging agents, but not to other classes of drugs upon telomerase inhibition in HeLa cells, and suggested a specific interaction between telomerase and the DNA repair process in human cells (11). However, this discrepancy may depend on the different cell lines used. Finally, we observed a similar behavior in response to drug treatment in MCF-7 and YCC-B2 cells with different p53 and estrogen receptor status, indicating that the dn-hTERT–dependent antiproliferative effects do not require functional p53-dependent checkpoints and could be achieved in both estrogen receptor–positive and estrogen receptor–negative breast tumor cells.

Previous studies have reported that in mouse cells lacking functional telomerase, treatment with anticancer drugs causes an increase in multichromosomal fusions and subsequently cell death, suggesting that telomere dysfunction due to telomerase inhibition, and not telomerase inhibition per se, is the most likely cause of increased drug sensitivity in those cells (44, 45). Although we did not detect any obvious increase in telomeric fusions or chromosome aberrations in cells expressing dn-hTERT without drug treatment, we have not characterized the cytogenetic profile of these cells after drug treatment. However, based on our data and the data reported in the literature, we speculate that shortened telomeres may be contributing to drug sensitization in the dn-hTERT derivatives. For instance, telomerase preferentially elongates the shortest telomeres (40, 41); thus, this group of telomeres may be more affected by the lack of telomerase activity upon dn-hTERT expression, and this might account at least in part for the higher drug sensitivity at early time points when most telomeres are still long.

In conclusion, our data show that telomerase inhibition in breast cancer cells cooperates with anticancer drugs to induce cell death and this effect depends on telomere shortening, although it does not require complete loss of telomeric sequences or functions. Rather, the presence of shorter telomeres may impair the ability of the cells to recover from drug treatment. The dependence on telomere shortening is supported by a recent observation that inhibition of telomerase by the chemical compound BIBR1532 (18) results in increased drug sensitivity only in

cells whose telomeres shortened in response to BIBR1532 and removal of this compound reverses both telomere shortening and the increased drug sensitivity (15).

Our results, together with those of other laboratories using other cell types, confirm that a combination strategy based on telomerase inhibition and anticancer drugs may be effective in inducing cell death of breast cancer cells.

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